COMBINATION IMMUNOTHERAPY TARGETING HSP90 DNA REPAIR CLIENT PROTEINS OVEREXPRESSED IN MELANOMA

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ABSTRACT

DNA repair protein (DNA-RP) overexpression in tumor cells has been reported to involve post-translational protein stabilization mediated by the molecular chaperone, Heat shock protein 90 (HSP90), thereby preventing proteasome-dependent degradation of these HSP90 "client" proteins. This overexpression of DNA-RP in a cancer-stage associated manner has also been correlated to chemotherapy resistance and poor overall prognosis. Hence, HSP90 inhibitors (HSP90i) have been heralded as cotherapy agents for cancer patients that have developed resistance to first-line treatments such as temozolomide (TMZ). Under conditions in which HSP90 function is blocked by HSP90i, HSP90 client proteins rapidly become polyubiquinated and undergo cytosolic degradation, resulting in sensitization of cancer cells to chemotherapy. We observed that HSP90i promote the proteasome-dependent degradation of a range of DNA repair client proteins, from which, we were able to define nine H-2K^b/D^b-presented peptide epitopes that could be recognized by Type-1 CD8$^+$ T cells after specific vaccination. When combined with an adoptive cell therapy (ACT) using anti-DNA-RP CD8$^+$ T cells, HSP90i-cotreatment yielded superior anti-tumor efficacy against TMZ-resistant B16 melanomas established in the dermis of syngenic C57BL/6 mice.

Innovation. This is the first study to focus on the use of HSP90i to conditionally promote the degradation and subsequent presentation of DNA-RP-derived peptides in MHC class I complexes, allowing for improved recognition of chemotherapy-refractory tumor cells by the adaptive (T cell) immune system *in vitro* and *in vivo*.

Public health significance. Melanoma is the deadliest form of skin cancer, with increasing incidence worldwide. This study defines a novel approach that may be translated into the clinic for the treatment of patients with advanced-stage melanomas or alternate chemotherapy-resistant forms of cancer. This approach could be readily combined with a range of synergistic immunotherapies as a novel first- or second-line treatment option for patients that have previously developed (genotoxic or targeted) therapy-specific resistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

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1.0 INTRODUCTION

1.1 MELANOMA OVERVIEW

Melanoma is the deadliest form of skin cancer. It has steadily increased in incidence worldwide over the past several decades. Even though it is not the most common type of skin cancer, melanoma contributes to approximately 75% of skin cancer-related deaths annually (Siegel et al., 2013; Siegel et al., 2016). Melanoma is a malignancy that evolves from melanocytes, skin cells that make the pigment, melanin. Melanoma often originates from a benign mole (also called a nevus) and can be identified by morphometric **a**symmetry, uneven **b**orders, the presence of more than one **c**olor in the lesion, a **d**iameter larger than ¼ inch and constant **e**volution over time; often referred to as the "ABCDE" rule for skin cancer diagnosis. A biopsy is recommended to be performed for diagnostic purposes on any suspicious lesion identified upon routine inspection of the skin (Guida et al., 2012). However, it is important to note that while most melanomas occur in the skin, there are instances for melanoma developing in mucosal surfaces and the uveal tract as well. In the past, melanomas have been divided into different subtypes based on their site of origin; i.e., cutaneous (skin), uveal (eye) and mucosal (lungs, gut, reproductive organs; Acquaviva et al., 2014). These three melanoma types differ in their etiology, geographic incidence distribution and clinical behaviors. Cutaneous melanoma occurs in the skin and is the most common type of melanoma. Cutaneous melanomas can be divided into four types based on histologic growth patterns; these are superficial spreading melanoma,

An alphabetical list of abbreviations is provided in Appendix A

nodular melanoma, acral lentiginous melanoma and lentigo melanoma. Uveal melanoma is a rare form of melanoma that occurs in the eye (Jewell et al., 2010).

Metastases of cutaneous melanomas can be segregated into three types based on their location relative to the primary lesion, i.) local metastasis, when a second lesion occurs within 2 cm of a primary tumor, ii.) regional metastasis, when the secondary lesion is farther than 2 cm from the primary tumor, but is in the same anatomic region of the body, and iii.) distant metastasis, where the secondary lesion is identified in a different region of the body than the primary tumor (Maverakis et al., 2015).

Despite melanoma representing one of the few cancers whose incidence has gone up dramatically over the last five decades, therapeutic advances capable of providing durable benefits to patients with melanoma remain limited. This increase in the incidence of melanoma and the lack of consensus curative treatment options for afflicted patients denotes a significant public health concern (Ha et al., 2011).

1.2 MELANOMA PUBLIC HEALTH OVERVIEW

UV exposure is a key risk factor in the development of melanoma, and geographic locations in which individuals reside are predictive of both the degree of UV exposure and relative melanoma incidence, with Australia currently having the highest melanoma incidence per capita in the world (Stecklein et al., 2012). Genetics also strongly influences the distribution of melanoma incidence. Lighter-skinned individuals have a much higher incidence rate of developing melanoma in comparison to their darker skinned counterparts, with a 3-7% annual increase in incidence of this disease amongst Caucasians (Che et al., 2013). The American Cancer Society

estimates that there will be 76,380 new cases of cutaneous melanoma diagnosed in 2016, with 10,130 deaths expected in the US from this disease. Melanoma is the 6th most common type of cancer in the US (at 4.5% of all new cancer cases reported in the US). Melanoma occurs more commonly in men than women overall, however, before age 50, incidence is higher in women than men. Strikingly, after age 65, the rates of melanoma incidence in men are double those observed for women, and by age 80, they are tripled for men vs. women. The gender-linked disease incidence profile may be due to differences in changing patterns of occupational and recreational exposure to ultraviolet radiation by men vs. women as they age. The median age at diagnosis for melanoma is 63 and the median age for death amongst those afflicted with this disease is 69. Notably, the earlier a person is diagnosed with melanoma, the better their chances for 5 year survival (Schmidt-Ullrich et al., 2003; Dote et al., 2006; Ko et al., 2012; Siegel et al., 2016).

1.3 CURRENT THERAPIES FOR MELANOMA

Common therapies for patients with melanoma that are currently applied in the clinic include surgery, radiation therapy, chemotherapy, targeted therapy and immunotherapy.

1.3.1 Surgery

When melanoma is localized and limited to a particular site of the body, surgery is an obvious first choice of treatment intervention. It is also used as a palliative treatment option when the cancer has metastasized to lungs or brain to relieve pain and to increase overall patient survival

(Younes et al., 2013). Surgery requires careful attention to disease margins and doesn't work well as a single modality when metastatic disease is suspected, since it is hard to identify and remove microscopic metastases. Surgery is usually used prior to targeted therapy or immunotherapy as a means to "de-bulk" patients and to minimize the absolute number of tumor cells that must be directly impacted by the chosen subsequent immunotherapy (Maverakis et al., 2015).

1.3.2 Radiation Therapy (RT)

Radiation is usually used as an adjunct therapy to alternate systemic therapies including cytotoxic chemotherapy, immunotherapy or combination approaches (Bhatia et al., 2009; Maverakis et al., 2015). Radiotherapy is a reasonable option for palliation; it was proved effective on melanoma patients with non-CNS metastasis, with complete resolution in 9% of cases and partial improvement in 75% of patients treated with a median dose of 30 Gy (Olivier et al., 2007).

1.3.3 Chemotherapy

Conventional cytotoxic chemotherapy has been used to treat melanoma for over 30 years. The two most commonly used chemotherapy drugs for melanoma patients are the alkylating agents, dacarbazine and temozolomide (TMZ) (Schindler & Postow, 2014). Although dacarbazine is FDA-approved for the treatment of melanoma, both agents seem to have similar efficacy with respect to patient overall survival, progression-free survival and quality-of-life benefit. Notably, TMZ has an additional benefit of being delivered orally, with the agent readily penetrating the

blood-brain barrier to truly provide systemic impact in treated patients. However, neither drug has been demonstrated to provide an overall survival benefit when evaluated in randomized clinical trials, requiring them to now be used in combination approaches (Middleton et al., 2000; Quirt et al., 2007).

1.3.4 Targeted Therapy

Activation of the mitogen-activated protein kinase (MAPK) pathway has become an oncogenic hallmark of melanoma, with approximately 50% of melanomas containing activating mutations. Activated MAPK pathway signaling leads to phosphorylation and activation of RAS, RAF, MEK and ERK, which impacts cellular differentiation, proliferation, survival and apoptosis of cells (Shah & Dronca, 2014). BRAF is commonly mutated amongst patients with melanoma (66% somatic missense mutations), with the BRAF(V600E) activating mutation most frequently detected amongst advanced-stage melanoma patients (Davies et al., 2002). This has made BRAF(V600E) protein an attractive therapeutic target in the melanoma setting. Vemurafenib, a specific synthetic small-molecule inhibitor of activated BRAF(V600E) has been evaluated extensively in clinical trials, with phase I/II trial results suggesting objective response rates (ORR) >50% for patients harboring BRAF(V600E) mutant melanomas. A phase III clinical trial comparing dacarbazine vs. vemurafenib in 675 melanoma patients with the BRAF(V600E) mutation supports the therapeutic superiority of vemurafenib based on extended overall survival. However, 38% of treated patients required dose de-escalation because of vemurafenib's toxic effects (Chapman et al., 2011). These favorable clinical findings led to FDA approval of vemurafenib for patients with BRAF(V600E) mutant melanoma in 2011 (Schindler & Postow, 2014). Dabrafenib, an alternate small-molecule BRAF inhibitor (BRAFi), with a similar activity

profile to vemurafenib, has also demonstrated clinical efficacy in patients with solid tumors, especially those with melanoma and untreated asymptomatic brain metastasis (Falchook et al., 2012). The single-agent MEK inhibitor, Trametinib has also shown clinical activity against BRAF mutant melanomas (Johnson et al., 2015).

However, despite such clinical benefits (with many patients exhibiting profound tumor regression), the vast majority of melanoma patients treated with BRAFi develop drug-refractory disease within 6-8 months of initiating treatment. Potential mechanisms of treatment-resistance include the activation of alternative oncogenic signaling pathways that reactivate downstream elements of the MAPK signaling pathway (Sullivan et al., 2013).

1.3.5 Immunotherapy – Checkpoint Indicators

Immunotherapy has been applied for the treatment of melanoma for decades, with high-dose interleukin (IL)-2 being FDA approved as a therapy option for patients with metastatic melanoma in 1998 (Maverakis et al., 2015). Recent years have seen an explosion in the clinical use of novel immune checkpoint inhibitors that prevent the tumor-associated senescence of therapeutic T cells in patients. Interestingly, immunotherapies have been shown to elicit more durable anti-tumor benefits in patients when compared to targeted therapies, such as those implementing BRAFi (Johnson et al., 2015). Ipilimumab is a monoclonal antibody reactive against the cytotoxic T lymphocyte antigen 4 (CTLA4) molecule expressed by regulatory T cells (Treg) and some previously-activated T effector cells. Anti-CTLA4 antibody blocks the down-regulation of T cell activation resulting from CTLA4-mediated signaling in Teff cells and limits the suppressive function of Treg, leading to overall enhancement of anti-tumor T cell effectiveness *in vivo* (Maverakis et al., 2015). In a phase III clinical study involving previously-

treated metastatic melanoma patients, Ipilimumab improved overall survival when applied as either a monotherapy or when combined with a vaccine (targeting the melanoma-associated antigen PMEL)(Hodi et al., 2010). In 2011, the FDA approved Ipilimumab for the treatment of metastatic melanoma patients (regardless of BRAF mutational status) (Niezgoda et al., 2015). Tremelimumab, an alternate CTLA4 blocking antibody, did not result in an improvement in overall survival when compared to standard chemotherapy in a phase III clinical study, suggesting significant operational variability in this class of anti-cancer agents (Ribas et al., 2013).

The programmed cell death 1 receptor (PD-1) is expressed by T effector cells. When T cell PD-1 is bound by its ligand PD-L1, which is upregulated on the cell surface of melanoma cells and tumor-associated macrophages (TAM), T cells are induced to undergo apoptotic cell death (Maverakis et al., 2015). Nivolumab and pembrolizumab are antagonist antibodies against PD-1 that have been recently evaluated as therapeutic agents in the melanoma setting. Patients with advanced-stage melanoma treated with nivolumab demonstrated improved median overall survival with high rates of tumor regression (Topalian et al., 2014). In another study, 28% of melanoma patients exhibited objective clinical responses after treatment with nivolumab (Topalian et al., 2012). In a phase I study combining nivolumab (anti-PD-1) with Ipilimumab (anti-CTLA4) in patients with advanced-stage melanoma, ORR were observed in 40% of patients treated with the combined regimen. At the maximum dose evaluated, 53% of treated patients had ORR, with over 80% of patients displaying some degree of reduction in tumor size (Wolchok et al., 2013). Interestingly, in a phase I study in which patients developed disease progression after receiving at least two doses of ipilimumab, such patients showed an overall response rate of 26% after subsequent treatment with pembrolizumab (Robert et al., 2014). These

findings suggest that combination therapies integrating multiple immune modulating agents may have the greatest potential to yield durable clinical benefits in cancer patients.

Table 1. Current Therapies for Melanoma.

Treatment modality	Drugs	Mechanism of action	Resistance mechanism
Chemotherapy	Dacarbazine Temozolomide	DNA-methylation	DNA-RP overexpression
Targeted therapy	Vemurafenib Debrafenib	BRAF V600E inhibitors	Activation of alternative oncogenic signaling pathways
	Trametinib	MEK inhibitor	
Immunotherapy	IL-2	Stimulation of T cell	Tumor escape from immune surveillance mechanisms
	Ipilimumab Tremelimumab	CTLA-4 blockade	
	Nivolumab Pembrolizumab	PD-1 blockade	

Current therapy options for patients with melanoma remain ineffective in providing durable ORR in the absence of profound toxicity when applied as single modalities, making the design and implementation of combination therapies a necessity [Adapted from: (Tentori et al., 2013)].

1.4 DNA DAMAGE RESPONSE

All of the cells in our body are susceptible to DNA damage as a result of environmental insults, but are protected in their normal somatic integrity based on the intrinsic action of DNA repair mechanisms (Hoeijmakers, 2009). For Single Stand Breaks (SSB) in genomic DNA, cells

employ repair mechanisms like Mismatch Repair (MMR), Base Excision Repair (BER) and Nucleotide Excision Repair (NER), while for more toxic double strand DNA breaks (DSB), cells employ mechanisms including Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) (Hoeijmakers, 2001). In addition to DNA repair mechanisms, the DNA damage response also includes cell cycle checkpoint regulation, transcriptional program activation, and regulation of cell death mechanisms. Improper functioning of these DNA repair pathways results in genetic instability, that in the absence of cellular lethality, may result in the development of cancers, such as cutaneous melanoma (Hoeijmakers, 2009; Warmerdam & Kanaar, 2010).

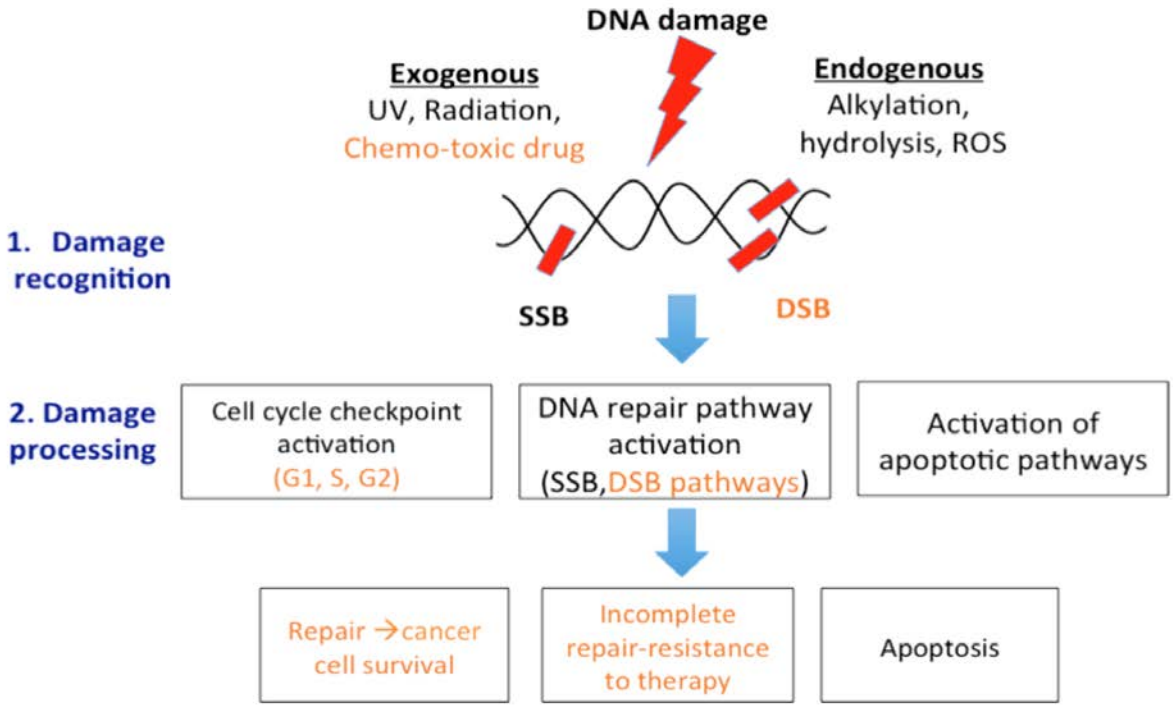


Figure 1. DNA Damage Response Mechanism.

The exogenous damage caused by chemotherapy drugs that induces toxic double strand breaks (DSB) is an issue of concern that results in outcomes of incomplete repair in cancer cells, which results in development of resistance to the chemotherapy [Adapted from: (Kinsella, 2009)].

1.4.1 Cell Cycle Checkpoint Regulation

There are three cell cycle checkpoints – G1-S, S phase and G2-M; at which the cell attempts to recognize and remove/repair DNA damage. Checkpoints operationally allow for a period of cell cycle arrest during which efficient DNA damage repair may be carried out before the onset of cellular mitosis or replication, when the aberrant (irreparable) cells may be induced to undergo apoptosis (Zhou & Elledge, 2000). In the G1 phase of the cell cycle, the cell has only one copy of DNA present and the major DNA repair pathway for DSBs during this phase is NHEJ. Affected cells respond to DNA damage through the ATM(ATR)/CHEK2(CHEK1)-TP53/MDM2 pathway (Kastan & Bartek, 2004; Hinz et al., 2005; Warmerdam & Kanaar, 2010). The G1-S checkpoint is TP53-dependent and is regulated by ATM (Massagué, 2004). Cells entering S phase with unrepaired DNA lesions are arrested in the S phase which is regulated by ATR being recruited to single strand DNA by the RPA binding complex in order to activate CHEK1 which signals cell cycle arrest. This prevents premature mitosis, chromatin condensation and mitotic apoptosis (Pavey et al., 2013). The G2-M checkpoint stops cells with DNA damage from initiating mitosis. ATM/ATR and CHEK1/CHEK2 inhibit the activation of the mitosis-promoting cyclin B/CDK1 kinase. The G2 checkpoint is partially maintained by BRCA1 and TP53 (Kastan & Bartek, 2004). Targeted interruption of these cell cycle checkpoints has been shown to sensitize cancer cells to the subsequent action of chemotherapeutic agents (Zhou & Bartek, 2004; Sankunny et al., 2014).

1.4.2 DNA Double Strand Break Repair

The DSB is the most dangerous form of DNA damage and its repair is crucial for cell survival and genomic integrity. The two main mechanisms through which DSBs are repaired are: error-prone NHEJ and an error-free mechanism based on HR between sister chromatids (Khanna & Jackson, 2001).

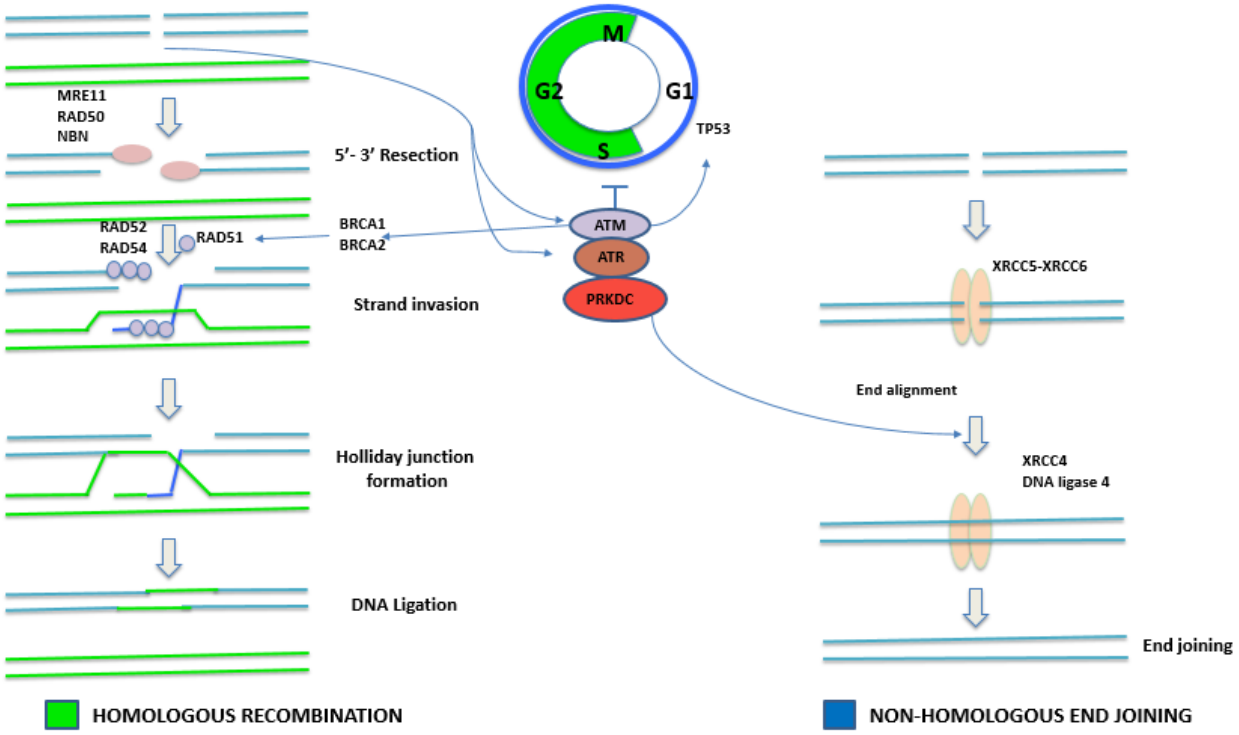


Figure 2. DNA Double Strand Break Repair.

Homologous recombination occurring via the error-free mechanism that predominantly occurs during the S or G2/M phase, and NHEJ, the error prone repair mechanism occurring at all phases of the cell cycle are the two major pathways used by cells to repair double strand breaks [Adapted from: (J. H. Hoeijmakers, 2001)].

1.4.2.1 Homologous Recombination (HR)

HR operates predominantly in the late S or G2 phases of the cell cycle, since it is dependent on a sister chromatid for use as a template for resynthesizing regions of DSB damage

(Shiloh & Lehmann, 2004; Kelley et al., 2014). The MRN complex, comprised of MRE11, RAD50 and NBN acts as a "sensor" (Williams et al., 2007) that initiates the resection at the 5' end on either side of DSB, creating a 3'-overhangs of single-strand DNA. ATM senses and may bind to the DSB, thereby activating/phosphorylating H2AX into γH2AX, which then attracts BRCA1 and NBN (Yuan et al., 2010; Sankunny et al., 2014). BRCA2 is recruited to the DSB by BRCA1, and facilitates the loading of RAD51 onto RPA-coated DNA overhangs with the help of RAD51 paralogs, which in turn, recruit RAD52 and RAD54. Accumulation of RPA leads to the activation of the ATR pathway (Shiloh, 2001). The tumor suppressor TP53, known to interact with BRCA1, RAD51, BLM and WRN, is also likely present within this DNA protein complex (Valerie & Povirk, 2003). The RAD51-coated single-stranded tail then searches for the homologous DNA sequence, and once identified, RAD51 mediates the strand invasion and successive ligation mediated by DNA ligase 1 to yield an intermediate structure known as a Holliday junction (Dexheimer, 2013; Kelley et al., 2014). There are then two possibilities to complete HR, either by non-crossing-over in which case the Holliday junctions disengage or by DNA strand pairing followed by gap filling (Hoeijmakers, 2001).

1.4.2.2 Non-Homologous End Joining (NHEJ)

NHEJ operates in all phases of the cell cycle in mammalian cells. The end-joining reaction in NHEJ simply links ends of a DSB together, without the need for a template, applying the end-binding XRCC5/XRCC6 complex to the exposed DNA termini of the DSB. After binding, the complex adopts a ring-shaped structure to encircle the DNA duplex. Once encircled, PRKDC is recruited to contact DNA termini (Walker et al., 2001). After the appropriate processing of the DNA termini, ligation is carried out by DNA ligase 4 in conjunction with XRCC4-Ligase 4 (Hoeijmakers, 2001; Dexheimer, 2013). The cell cycle machinery, including

the DNA damage signaling protein kinases ATM and ATR, influences the DSB repair pathway choice (Hoeijmakers, 2001).

1.4.3 DNA Repair Proteins as Targets for Immunotherapy

Despite decades of research, and in spite of the recent advent and success of immune checkpoint blockade-based immunotherapeutic interventions, until recently, the most commonly applied treatment for melanoma (and indeed most forms of cancer) was chemotherapy (CT). In order to clinically reduce tumor size, CT causes “incorrectable” DNA lesions in treated tumor cells, most commonly DSBs (Fojo, 2001; Luqmani, 2005; Khan et al., 2011). Moderately impacted tumor cells amplify DNA repair mechanism associated gene products in order to maintain viability and foster a state of CT-resistance (Kauffmann et al., 2008). In order to prevent such resistance and to increase melanoma sensitivity to CT, we chose to develop a novel immunotherapy approach targeting DNA repair proteins that have been highly-correlated with the progression of drug-refractory disease after CT (Kauffmann et al., 2008).

In particular, since CT may promote elevated expression of DSBs, critical DNA-RP in the HR and NHEJ pathways involved in DSB repair for focused immunologic evaluations were chosen; i.e., ATM, ATR, BRCA1, BRCA2, CHEK1, CHEK2, PRKDC; XRCC6, XRCC5, MRN complex proteins (MRE11, NBN, RAD50) and RAD51 (Shiloh & Lehmann, 2004; Kauffmann et al., 2008; Stingl et al., 2012).

1.5 ROLE OF HSP90 IN CANCER

Heat shock protein 90 (HSP90) is a highly-conserved molecular chaperone that is essential for the stability and proper folding of its client proteins. HSP90 helps these client proteins regain their native conformations and sustains their functionality under conditions of severe cellular stress. Many HSP90 client proteins play important roles in pro-cancer cellular processes, such as angiogenesis, metastasis, cell signaling, cell proliferation and cell survival. HSP90 aids cancer cells by protecting the activity of pro-tumor client proteins by refolding and stabilizing them in an environment that is not conducive to normal metabolic programming (i.e., the hypoxic microenvironment in tumors). This makes HSP90 an important target for the development of effective interventional cancer therapies.

1.5.1 Heat Shock Proteins

Heat shock proteins were first discovered more than 50 years ago when a puffing pattern was observed in the chromosomes of *Drosophila* under elevated temperature (Ritossa, 1962). By definition, heat shock proteins (HSPs) are proteins whose expression is elevated when the temperature increases above a physiologic norm. Expression of HSPs is also upregulated in response to other cellular stressors, including hypoxia, acidosis and physical pressure (De Maio, 1999). HSPs serve as chaperones in helping other proteins recover from misfolding events that would typically inactivate those proteins and lead to their degradation via the macromolecular, multi-enzymatic proteasome complex (Blagosklonny, 2002). HSPs support a highly-conserved and primitive molecular salvage pathway that optimizes the likelihood of survival of cells under pathologically stressful conditions.

HSPs are highly abundant proteins (1-2% of the total proteome) in normal cells, with even higher levels (up to 5-6%) detected in tumor cells. HSPs regulate the fate of thousands of intrinsic cellular proteins by facilitating the ATP-dependent refolding and reassembly of multiprotein complexes in the cytosol, endoplasmic reticulum and mitochondria, and are also involved in the intracellular transport and sorting of proteins (Buchner et al., 1999). HSPs consist of a large family of proteins that are individually classified based on their molecular weights; i.e., HSP10, HSP27, HSP40, HSP60, HSP70, HSP90, etc. (Li & Srivastava, 2004).

1.5.2 Heat Shock Protein 90 (HSP90)

Heat shock protein 90 is a highly-abundant, stress-inducible, homodimeric, ATP-dependent molecular chaperone. In normal cells, HSP90 exists as a free protein and its chaperone activity is modest. On the other hand, in cancer cells, HSP90 is frequently overexpressed and plays an important role in the maintenance of protein conformational integrity, stabilization of a number of oncogenic/survival proteins, supporting a cytoprotective response to the hypoxic and acidic tumor microenvironment (TME). In such a setting, HSP90 supports tumor progression and metastasis (Whitesell & Lindquist, 2005). In tumor cells, HSP90 forms the core of a super chaperone machine, consisting of HIP, HOP, HSP40 and HSP70, which have been shown to maintain the functional integrity of a growing list of client proteins, including signaling protein kinases, transcription factors, DNA-RP and other cytosolic or nuclear proteins (Taipale et al., 2010). HSP90 client proteins are involved in each of the original six hallmarks of cancer cell physiology i.e., self-sufficiency in growth signal, insensitivity to growth-inhibitory mechanism, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg, 2000; Barrott & Haystead, 2013).

Most chemotherapeutic agents and small molecule inhibitors were selected for use based on the biased targeting of only one to two individual proteins or signaling mechanisms. Given the striking molecular heterogeneity of the aggregate tumor cell population in a given patient, such targeting predictably selects for tumor cell subpopulations that exhibit functional compensatory pathways that are refractory to the action of a given targeted treatment regimen. In contrast, the therapeutic targeting of HSP90 would theoretically lead to the coordinated disruption of multiple pathways supporting cancer survival and metastasis (Koga et al., 2009), reducing the odds for outgrowth of treatment-refractory disease. Additionally, since HSP90 is highly overexpressed in tumors, HSP90 targeting is more selective against cancer (versus normal cells)(Kamal et al., 2003). Therefore, inhibition of HSP90 has gained substantial traction as a novel cancer therapeutic strategy.

1.5.3 HSP90 Structure & Function

The HSP90 family consists of the cytosolic HSP90-α and HSP90-β, the ER HSP90B1 and the mitochondrial TRAP1 (Ciocca et al., 2013). In humans, HSP90-α and HSP90-β are closely-related isoforms, that both become activated under cellular stress conditions(Csermely et al., 1998; Goetz et al., 2003). HSP90 is a phosphorylated homodimer which contains two to three phosphate groups (Buchner et al., 1999). Each monomer in the homodimer consists of a highly conserved amino-terminal domain, a middle domain, which binds to HSP90-client proteins, and a carboxy-terminal domain that helps in dimerization. HSP90 has a highly-conserved ATP-binding domain near the amino-terminal domain. This ATP-binding domain is comprised of an α- and β-sandwich motif (Taipale et al., 2010). After interacting with client proteins and co-chaperones, binding and hydrolyzing of ATP takes place at the ATP-binding domain (Buchner et

al., 1999). The HSP90 dimer is a flexible structure due to the antiparallel association of the component C-terminal regions. The relative orientation of the domains allows HSP90 to adopt various conformations. A rise in temperature leads to the formation of a closed conformation by triggering an additional structural transformation that brings the N-terminal domains into close proximity (Barrott & Haystead, 2013). ATP binding to HSP90 leads to a "closed" conformation. Hydrolysis of ATP into ADP leads to a secondary state that remains structurally unresolved, with subsequent dissociation of ADP dissociation restoring HSP90 to its open conformation. The closed conformation in the ATP-bound state is the active form of HSP90, enabling this molecule to optimally function within the multi-component chaperone complex (Ciocca et al., 2013; Taipale et al., 2010).

HSPs are regulated primarily at the transcriptional level by heat shock factor-1 (HSF1). HSF1 is constitutively expressed in cells (Ciocca et al., 2013). HSF1 is usually bound to HSP90 and is inactive, however, under stressful conditions, HSF1 is released from HSP90 and becomes phosphorylated by protein kinases, which results in the formation of homotrimers that enter the nucleus and then bind to heat shock elements in the promoter region of the HSP90 gene and other HSP genes. HSP90 mRNA is transcribed after further phosphorylation and enters the cytosol where new HSP90 chaperone complexes are then assembled (Buchner et al., 1999; Richter & Buchner, 2001).

Co-chaperones assist HSP90 throughout its conformational cycling, which is essential for optimal function, acting as substrate recognition proteins and even enhancing enzymatic activity. The tetratricopeptide repeat (TPR) domain containing proteins are the predominant class of co-chaperones, which bind the MEEVD motif located in the C-terminus of HSP90. HSP70–HSP90 organizing proteins (HOP) are co-chaperone proteins that interact with the C-terminus of HSP70.

Other co-chaperones that interact with HSP90 through alternative domains are activator of HSP90 ATPase homolog 1 (AHA1), which augments the function of HSP90 by stimulating its ATPase activity. Cell division cycle 37 (CDC37) is the co-chaperone that is most implicated in facilitating tumorigenesis, as it associates with mutant kinases that drive cancer progression (Stepanova et al., 1996). P23 is another co-chaperone that is responsible for the complexing of HSP90 and nuclear hormone receptors that has been found in a wide range of HSP90–client complexes. Unlike the activating co-chaperone AHA1, the co-chaperones CDC37, P23 and HOP inhibit ATPase activity. These co-chaperones therefore add an additional layer of regulation to this multifaceted master chaperone, underlining the complexity of the HSP90 chaperone cycle and its client protein salvaging functions (Buchner et al., 1999).

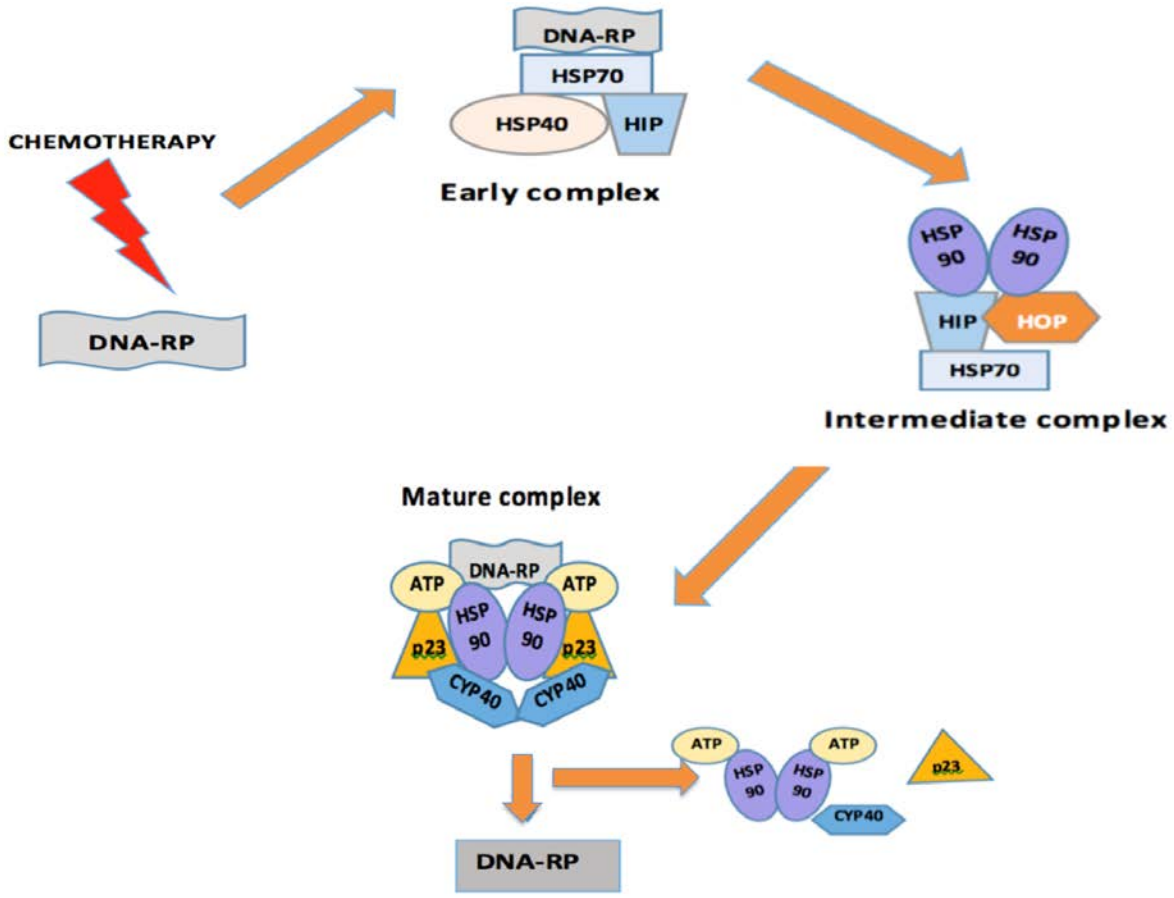


Figure 3. The HSP90 Superchaperone Salvages DNA-RP Clients.

HSP90 along with other molecular chaperones forms the mature complex capable of binding to client proteins (that have lost their structural and functional integrity in the stressful TME) and mediating their return to native conformation in support of cancer progression. Though this project focuses on the immune targeting of DNA-RP that represent HSP90 client proteins in cancer cells, the core paradigm for this work is applicable to all tumor-associated client proteins of HSP90 [Adapted from: (Whitesell & Lindquist, 2005)].

1.5.4 HSP90 Client Proteins

HSP90 has an ever-increasing number of known cellular client proteins. (For a comprehensive list of HSP90 client proteins, see http://www.picard.ch/downloads/Hsp90interactors.pdf). In the

absence of HSP90-mediated stabilization, most unfolded client proteins undergo degradation. In tumor cells, mutated proteins that may represent oncogenes (such as BRAF(V600E)) may be unstable under stress conditions, but are retained/accumulated in cancer cells due to the corrective action by HSP90 (Blagosklonny et al., 1996). HSP90 stabilizes oncoproteins such as BCR/ABL1, RAF1, ERBB2 and mutant forms of TP53; survival-signaling kinases such as AKT1 and PI3K; growth factor receptors such as VEGFA, PDGFRB and less stable proteins produced by DNA mutations (Fulda et al., 2010). A few of the protein kinases that play an important role in cancer progression are listed here. ERBB2 binds to HSP90 and to its endoplasmic reticulum homolog HSP90B1. It is a receptor tyrosine kinase that is overexpressed in many malignancies such as breast, prostate, gastric, and ovarian cancers. AKT1 is another protein kinase that controls cell proliferation and survival pathways and it therefore plays a role in cancer progression as it suppresses apoptosis. The inhibition of HSP90 using STA9090 results in the disassociation of CDC37 and CDK4, and a resultant reduction in the half-life of the CDK4 protein. RAF1 protein kinase is associated with an HSP90 complex containing CDC37 and HSP90N. The BCR–ABL1 fusion protein P210 whose upregulation is associated with CML is also an HSP90 client protein. (Buchner et al., 1999) Steroid hormone receptors require HSP90 to maintain the appropriate receptor conformation for hormone binding. The most commonly mutated tumor suppressor product, TP53, requires HSP90 to achieve a stable conformation (Trepel et al., 2010). Furthermore, a transcription factor and HSP90 client protein important to oncogenesis is Hypoxia-inducible factor-1α (HIF1A), which plays a role in cancer progression by supporting tumor-associated glycolysis, glucose transport and angiogenesis (Zhang et al., 2005).

The pro-apoptotic kinase, ASK1 (apoptosis signal-regulating kinase 1) loses its activity as a results of a complex formation with HSP90-AKT1 (Neckers et al., 1999). HSP90 exerts anti-apoptotic functions inside the mitochondria by interacting with TRAP1 (tumor necrosis factor receptor-associated protein 1), CYPD (cyclophilin D) and survivin. This also prevents AIF (apoptosis-inducing factor) mitochondrial-cytosolic translocation and inhibits the nucleolytic activities of both AIF and endonuclease G (Trepel et al., 2010). HSP90 also aids in cancer progression by promoting angiogenesis and metastasis through the chaperoning of specific client targets, including VEGFRs and PDGFRs, amongst others. Therefore, inhibition of HSP90 function in the TME is expected to exert a range of biologic effects that are largely contraindicated for tumor cell survival/cancer progression (Sangster et al., 2004).

HSP90 plays a crucial supportive role in tumorigenesis, as it is associated with a wide range of client proteins that are involved in tumor cell growth and metastasis. However, our understanding of HSP90 expression and function in various cancers continues to be incomplete. Much remains to be learned with regards to HSP90 activity in tumor cellular processes such as energy metabolism, protein trafficking, epigenetics and DNA quality-control. Many client proteins that play a role in DNA-damage response have been identified (Schmidt-Ullrich et al., 2003; Dote et al., 2006; Jewell et al., 2010; Stecklein et al., 2012; Guida et al., 2012; Ha et al., 2011; Ko et al., 2012; Che et al., 2013; Acquaviva et al., 2014; Pennisi et al., 2015) and are listed in Table 2. To aid in future studies of HSP90 functional impact in the cancer setting and to serve as possible therapeutics, many HSP90 inhibitors (HSP90i) have been developed over the past decades.

Table 2. HSP90 DNA Repair Client Proteins.

HSP90 Client proteins	HR	NHEJ	CELL CYCLE CHECKPOINT	FANCONI ANEMIA	BASE EXCISION REPAIR	NUCLEOTIDE EXCISION REPAIR	MISMATCH REPAIR
ATR	●	●	●				
BRCA1	●			●			
BRCA2	●			●			
CHK1			●				
CHK2			●				
PRKDC		●					
MRE11	●						
RAD50	●						
NBN	●						
ERCC1	●			●			
XRCC1		●			●	●	
PCNA					●	●	●
FANCD2				●			
MSH2							●
TP53			●				

HSP90 has several client proteins that play a role in the DNA damage response, with many of these proteins playing roles in more than one particular pathway. Overexpression of these proteins in cancer cells has been correlated with chemo resistance. However, the impact of HSP90i on the tumor cell-expressed components of the HR and NHEJ pathways remain unknown [Adapted from: (Dietlein et al., 2014; Pennisi et al., 2015)].

1.5.5 HSP90i

Since HSP90 is an ATP-dependent chaperone machine, drugs that interact with ATP-binding sites of kinases have been tested for their ability to inactivate HSP90 chaperone activity. Galdanamycin (GA), the first identified inhibitor of HSP90 chaperone activity, was originally thought to function solely as a kinase inhibitor, but then later found to be a HSP90 inhibitor that

could have clinical benefit. (Neckers & Ivy, 2003). Many GA-derived HSP90 inhibitors (HSP90i) were developed that were classified as first generation inhibitors. However, the first generation of inhibitors displayed substantial toxicity in cancer patients. The second-generation of inhibitors developed still required intravenous administrations on a weekly basis, but had greater bioavailability and exhibited less hepatic toxicity. Second-generation inhibitors are taken orally and have been demonstrated to be more stable, less toxic and exerted greater anticancer properties. There are currently 17 different HSP90 inhibitors that have been tested in clinical trials involving over 1000 cancer patients (Proia & Kaufmann, 2015; Trepel et al., 2010).

The impact of HSP90i on the tumor cell-expressed components of the HR and NHEJ pathways still remains unknown. However, previous reports have identified several DDR proteins including ATR, CHEK1, BRCA2, PRKDC and the MRN complex as client proteins of HSP90 (Schmidt-Ullrich et al., 2003; Dote et al., 2006; Jewell et al., 2010; Ha et al., 2011; Stecklein et al., 2012; Guida et al., 2012; Ko et al., 2012; Che et al., 2013; Acquaviva et al., 2014; Pennisi et al., 2015). Notably, in all of these studies, investigators focused on just one, or only a small subset of protein targets.

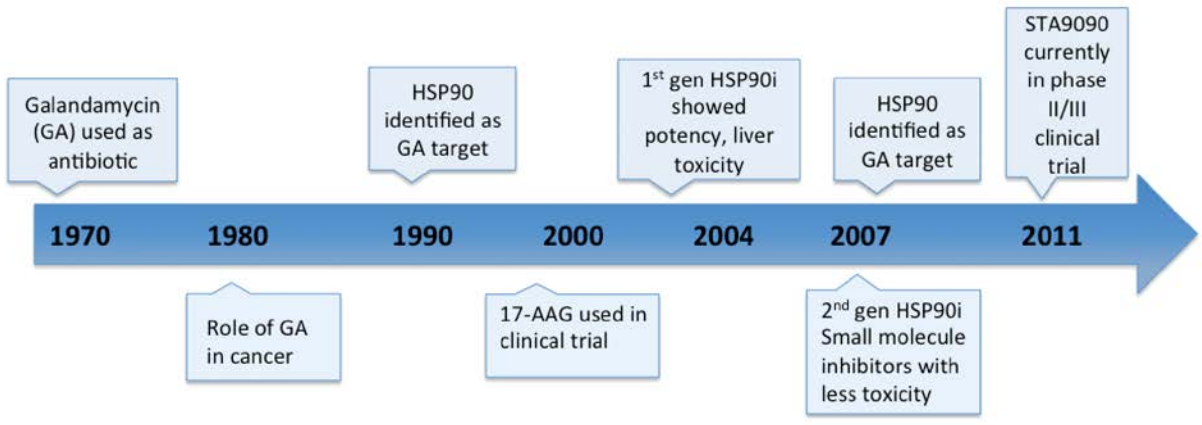


Figure 4. HSP90 Inhibitor Developmental Timeline.

GA was the first drug that was identified as an HSP90 inhibitor. Many GA-derived HSP90 inhibitors classified as 1st generation HSP90i showed modest potency in phase I clinical trials but had significant cardiac and liver toxicities associated with them. The 2nd generation inhibitors addressed the toxicity issues and also exhibited higher potency. STA9090, in particular had shown promising results in phase I/II clinical trials and is currently in a phase III clinical trial for the treatment of patients with NSCLC.

1.5.5.1 STA9090

STA9090 (aka, Ganetespib) when compared to the 1st generation drug 17-AAG, exhibits higher competitive binding to the ATP binding site at the N-terminal domain of HSP90 (Soga et al., 2013). This shifts HSP90 from a refolding chaperone complex to a form that promotes client proteins polyubiquitination and subsequent degradation by the 26S proteasome (Ha et al., 2011). STA9090 has shown promising results in both preclinical and phase I/II clinical trials in the setting of melanoma, metastatic breast cancer and NSCLC. STA9090 appears to work better when applied in combination rather than as a single modality drug, as may be the case for other previously investigated HSP90i (Proia et al., 2012; Socinski et al., 2013; He et al., 2014; Jhaveri et al., 2014).

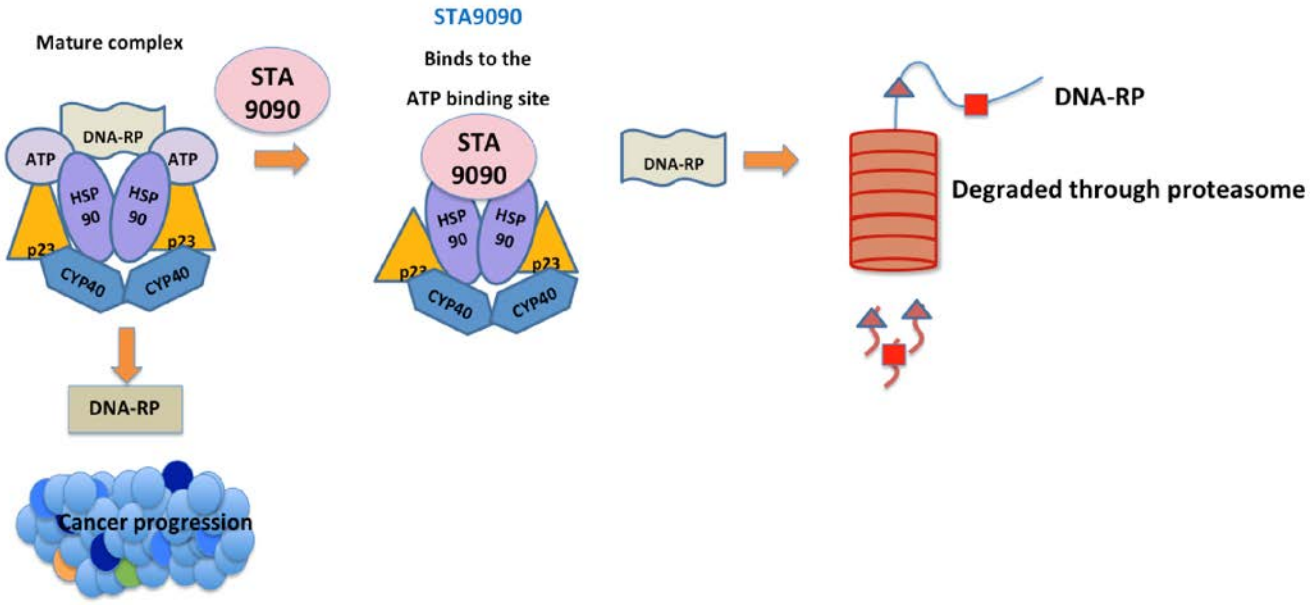


Figure 5. STA9090 Degrades Client Proteins through a Proteasome-Dependent Processing Pathway.

STA9090 prevents the HSP90 mature complex from binding to the client DNA-RPs by binding to the ATP site instead. Unchaperoned DNA-RPs client proteins then get degraded through a proteasome-dependent pathway, leading to increased sensitivity of tumor cells to the chemotherapeutic agents.

1.5.6 Combinational Anti-Tumor Therapy Integrating HSP90i

When applied as a single modality, HSP90i have yielded only modest anti-tumor efficacy and discernable toxicity. However, combination therapies integrating HSP90i in concert with chemotherapy and/or radiotherapy (CRT) have yielded encouraging results, with HSP90i pre-treatment appearing to sensitize tumor cells to subsequent CRT (to be discussed in more detail in Chapter 3). No studies have focused on the ability of HSP90i to alter immune reactivity against HSP90 client DNA-RPs, which speaks to the novelty of the current project.

1.6 CANCER IMMUNOTHERAPY

1.6.1 Cancer Immunity

The immune system has the ability to distinguish self from non-self cells and to destroy such invaders. Tumor immunology is somewhat more complicated, in that the host immune system must be able to differentiate tumor cells from normal cells based on antigens that are uniquely (i.e., mutated) or differentially (based on higher levels of protein) expressed by the diseased cells (Melvold & Sticca, 2007; Finn, 2008). In particular, host T cells can only be triggered to react to target cells if the level of cognate peptide presented in the context of MHC class I or class II molecules exceeds a threshold established as a consequence of "negative" selection during the neonatal self-tolerance programming of the host's T cell repertoire (Grossman and Paul, 2015). An improved understanding of the tumor antigens recognized by the T cells and the specific peptide sequences presented in MHC molecular complexes has helped to improve the efficacy and monitoring of immunotherapies over the past 25 years (Harris et al., 2013).

In 1991, van der Bruggen and colleagues were the first to use expression cloning techniques to identify a melanoma antigen recognized by $CD8^+$ T cells, which they termed MAGEA1 (Bruggen et al., 1991). Since then, over 100 melanoma-associated tumor antigens recognized by T cells have been defined (Yang, 2011). The tumor associated antigens described for melanoma can be mainly divided into four major classes. 1) Differentiation antigens, such as the melanocyte-specific proteins like MLANA, MSH1, Tyrosinase and tyrosinase-related proteins; 2) Embryonically-expressed proteins, also known as cancer-testis antigens like the MAGE family of proteins and CTAG1B; 3) Tumor-specific mutated proteins that produce immunogenic epitopes that are naturally-processed and -presented, as perhaps best exemplified

by CTNNB1 and CDK4 (Kawakami et al., 1996); and 4) Tumor overexpressed proteins that support the malignant phenotype or transformation process. These latter proteins can have important functions in normal tissues, as in the case of wild-type TP53, which is frequently overexpressed in cancer cells due to pairing with a mutated *TP53* allele (Cohen et al., 2005; Yang, 2011). The DNA-RP overexpressed in melanomas that are responsive to upregulation after chemotherapy would fall into category 4 above. This project represents a first attempt to investigate tumor overexpressed proteins involved in DSB pathway as cancer-specific antigens that can be targeted by T cells.

1.6.2 Cancer Immunotherapy

The concept of cancer immunotherapy is to use a patient's immune system to recognize, attack and reject their own malignant cells (Rosenberg et al., 2004). Several different approaches have been used to achieve this goal. By administering cancer vaccines, a patient's own immune system is educated to expand T cells that are capable of recognizing and killing their own tumor cells. Similarly, therapeutic antibodies (reactive against tumor cell surface antigens) or certain cytokines may be applied to facilitate immune cell-mediated destruction of tumor cells. Adjuvants (including cytokines, toll-receptor ligands, and even purified or recombinant HSP90) can be used to activate and mature endogenous patient Dendritic Cells (DC), Natural Killer Cells (NK cells), and Cytotoxic T Lymphocytes (CTLs) to mediate heightened anti-tumor immunity (Waldmann, 2003; Rosenberg et al., 2004). Amongst the myriad of cancer immunotherapy approaches developed over the past 50 years, adoptive cell therapy (ACT) and monoclonal antibody therapies targeting immune checkpoint molecules such as CTLA-4 and PD-1/PD-L1 have emerged as preferred treatment modalities (Rosenberg et al., 2008).

1.7 ADOPTIVE CELL THERAPY

Adoptive cell therapy is personalized cancer therapy that has emerged as an effective treatment for metastatic melanoma patients. ACT involves the generation of anti-tumor T cells from the blood or tumor of a cancer patient, with subsequent expansion of T cells *ex vivo* with subsequent reinfusion of these cytotoxic immune effector cells back into the same patient (Johnson et al., 2006; Rosenberg et al., 2008).

1.7.1 Tumor-Infiltrating Lymphocyte (TIL) Therapy

The typical procedure for TIL therapy involves the process of TIL expansion where resected tumor is cut into small fragments that are cultured in the presence of high-doses of IL2, with the tissue-resident TIL expanding for several weeks (using a so-called Rapid Expansion Protocol (REP) using anti-CD3 in the presence of antigen-presenting cells along with rIL2) before being reintroduced into the cancer patient by i.v. infusion (Wu et al., 2012). Studies have shown that TILs present in tumor tissues play an important role in both cancer progression and cancer treatment (Liao et al., 2013). The identification of the REP, enabling generation of large numbers (i.e., 10^9-10^{12}) of T cells was key to the development of adoptive cellular therapy; (Muul et al., 1987; Rosenberg et al., 2008). Clinical trials for metastatic melanoma have shown that the combination of TIL ACT with systemic administration of rIL2 results in tumor regression in 50 to 70% of treated patients (Dudley et al., 2008). There have been clinical trials performed using "young TIL" without implementation of a REP. This technique results in more TIL and an accelerated schedule to treat most enrolled patients. The results of this protocol suggest that lympho-depleting chemotherapy followed by young TIL ACT may lead to tumor regression in

50% of advanced-stage melanoma patients with manageable toxicities (Besser et al., 2010). In an alternate study using young TIL and NMA, 58% of advanced-stage melanoma patients exhibited objective clinical responses (OCR), while treatment with young TIL and 6 Gy irradiation yielded 48% OCR, with 4% complete responses (Dudley et al., 2010).

1.7.2 Genetically Engineered T Cell Therapy

Since it is not possible to obtain tumor-reactive TILs from all cancer patients for the purpose of delivering ACT, there has evolved the need to achieve this goal by genetically-modify large numbers of autologous T cells to exhibit anti-tumor specificity (Zhou & Zhou, 2014). In this process, bulk patient T cells are infected with a recombinant retroviral vector encoding either a tumor-specific T-cell receptor (TCR) or a chimeric antigen receptor (CAR) based on an antibody reactive against a tumor cell surface antigen (Rosenberg et al., 2008; Zhou & Zhou, 2014). CARs differ from TCRs as they target native cell surface antigens that do not require peptide processing or HLA-presentation, whereas, TCRs engage specific HLA-peptide complexes that need to be matched with a cancer patient's MHC haplotype (Sadelain et al., 2013). TCR-based ACT therapies targeting CTAG1B have led to tumor regression in patients with metastatic synovial cell sarcoma and melanoma, with 18% of patients with melanoma exhibiting complete tumor regression that persisted for over 1 year (Robbins et al., 2011). While there are several studies reporting clinical efficacy using CAR-T cell therapy targeting CD19 in acute lymphoblastic leukemia, chronic lymphoblastic leukemia, multiple myeloma and lymphoma (Dai et al., 2016), these are mostly limited to the treatment of hematological malignancies. In the end though, the anti-tumor impact of CAR-T cell-based therapy in solid tumors may be limited due

to the more suppressive TME of solid cancers and due to the well-known antigenic heterogeneity of the aggregate target cell population (Khalil et al., 2016).

1.8 MHC CLASS 1 PRESENTATION PATHWAY

Over the last 25 years, the necessity of MHC class I expression for the presentation of antigens to cytotoxic T lymphocytes has been made clear. The MHC class I antigen presentation pathway involves the processing of endogenous self or viral proteins for presentation of their peptides in the context of MHC class I molecules that are expressed on the surface of all somatic, nucleated cells in the body (Ramirez & Sigal, 2004). MHC class I molecules present peptides that are approximately 8-12 amino acids in length to specific subpopulations of $CD8^+$ T cells. This allows $CD8^+$ T cells circulating in the blood system to police peripheral tissues in search of aberrant infected or diseased cells, and to eradicate them in defense of the health of the host organism. Indeed, MHC class I-restricted cytotoxic T lymphocytes (CTL) responses are critical for effective anti-tumor immunity (Overwijk et al., 2003). MHC class I-restricted peptide presentation occurs via two distinct pathways, the direct- and cross-presentation pathways. Due to defects in MHC molecule expression or antigen processing machinery, and/or a skewed balance towards co-inhibitory over co-stimulatory molecule expression, tumor cells are generally considered to be poor antigen presenting cells (APC) to T cells (Storkus et al., 2007). Rather, T cell responses are elicited efficiently by professional APC, such as dendritic cells, through antigen cross-presentation to initiate a protective CD8+ T cell (including CTL) responses against tumor cells.

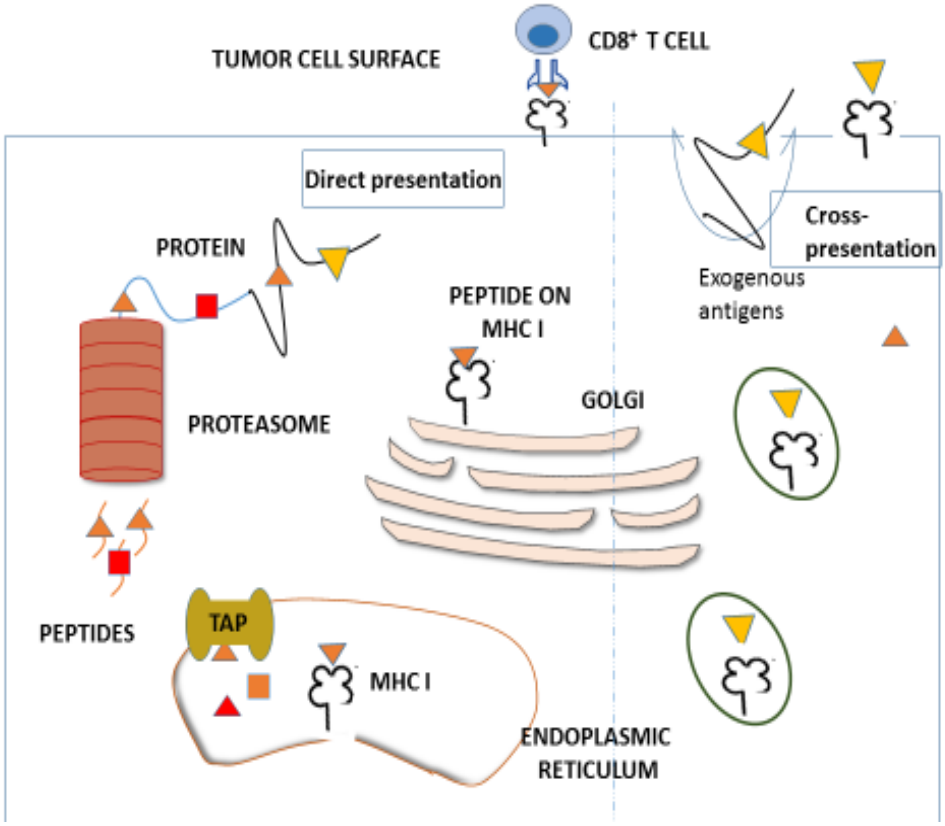


Figure 6. MHC Class I Pathway.

Proteins are processed by the proteasome in the cytosol and produce peptides and are translocated into the endoplasmic reticulum by the TAP1/TAP2 complex in an ATP-dependent manner. MHC class I-restricted peptide presentation occurs via two distinct pathways, the direct- and cross-presentation pathways before the peptides are transported to the surface for presentation [Adapted from: (Villadangos & Schnorrer, 2007)].

In the direct presentation pathway, intrinsic unfolded tumor-associated proteins such as ERBB2 and mutant BRAF, among others, may be retro-translocated into the cytoplasm via a SEC61A1-dependent mechanism, where they may be degraded by the multi-catalytic proteasome complex. Peptides, 8-10 amino acids in length, may be translocated from the cytosol into the ER by the transporter of antigenic peptide (TAP)-1/-2 molecules for binding in nascent MHC class I complexes (Heath et al., 2004). Longer peptides that are sufficiently stable in their interactions with MHC class I molecules enter the ER and may also be trimmed by an ER-resident aminopeptidases (ERAP1) (Elliott, 2006).

In the cross-presentation pathway, exogenous antigens are internalized by APC vi phagocytosis, endocytosis, pinocytosis or micropinocytosis. This cross-presentation pathway may compete with the MHC class II presentation pathway for exogenous antigens. Cross-presented antigens are transferred to the cytosol through a mechanism similar to ERAD (degradation of newly-synthesized and misfolded proteins), followed by proteasome-mediated degradation and transport into the ER by TAP, before final loading into newly-formed MHC-I complexES (Ramirez & Sigal, 2004; Villadangos & Schnorrer, 2007).

HSPs also contribute to the function and orchestration of the antigen-processing and MHC-presentation machinery of tumor cells which directly impact the ability of these aberrant cell populations to be recognized and regulated by protective T effector cells (Binder & Srivastava, 2005; Zhou & Binder, 2014). Furthermore, HSP90 plays a critical role in antigen cross-presentation, in which DCs bind to extracellular antigens, internalize them in endosomes, and then translocate them from endosomes into the cytosol for degradation by the proteasome (Imai et al., 2011).

Notably, HSP90i have been reported to enhance tumor cell MHC molecule expression and/or tumor antigen-derived peptide presentation by MHC I/II molecules on the surface of tumor cells, thereby facilitating the ability of moderate avidity T cells to recognize and react against treated tumor cells vs. untreated tumor cells or normal cells (Haggerty et al., 2014; Kawabe et al., 2009; Lin et al., 2007). Such conditional HSP90i-dependent alterations in the MHC-presented “peptidome” allow at least certain species to exceed the operational tolerance threshold of the host’s T cell repertoire, allowing for the activation and mobilization of a previously silent T effector cell cohort with potential to mediate anti-tumor activity. Modulations in tumor immunogenicity induced by interventional drugs such as HSP90i becomes even more

important clinically given pervasive reports for MHC and antigen-processing/presentation abnormalities in heterogeneous cancer cell populations *in vivo* that correlate with low levels of MHC-peptide complexes on the tumor cell surface, and with disease progression and poor patient survival (Lampen & Hall, 2011; Leone et al., 2013; Seliger, 2014).

Once on the cell surface, these MHC class I/peptide complexes may be surveyed by host $CD8^+$ T cells, some of which may mediate tumoricidal function (i.e., Type-1 $CD8^+$ T cells). For the purposes of this study, we propose that HSP90i (such as STA9090) can transiently bias MHC class I occupancy towards peptides derived from HSP90 client proteins involved in DNA repair in order to facilitate recognition by protective, antigen-specific $CD8^+$ T cells.

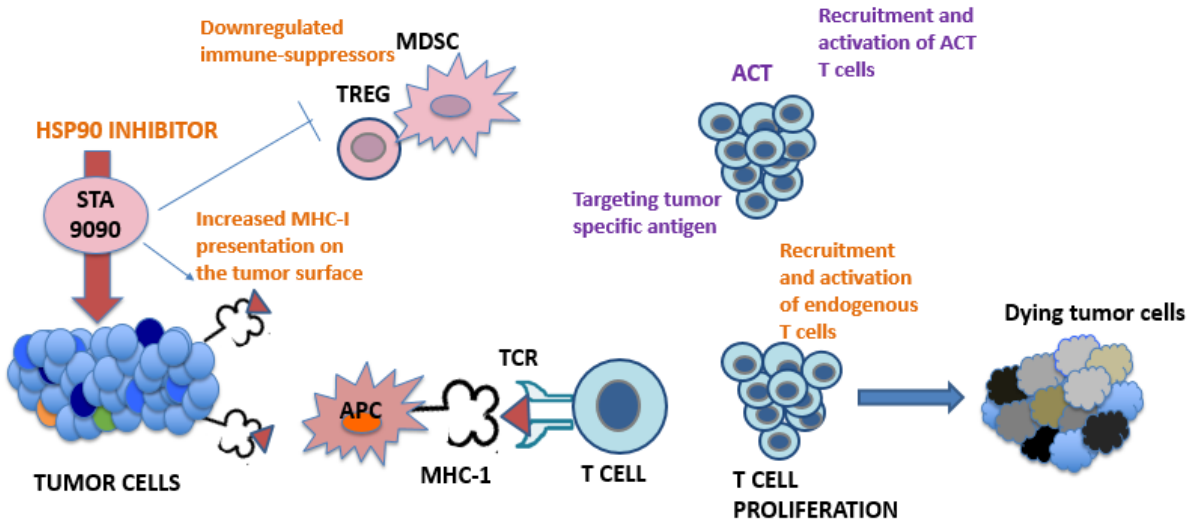


Figure 7. Rationale behind Combining HSP90i with Adoptive Cell Therapy.

In addition, previous work by our group suggests that HSP90i can coordinately reduce levels of immunosuppressive cell populations in the TME, including MDSC and Treg. The ability of HSP90i to promote enhanced antitumor activity from T effector cells (via ACT), while removing regulatory immunity in the TME supports the expectation for the superior antitumor efficacy of combined HSP90i + ACT approaches in the cancer-bearing host.

1.9 PUBLIC HEALTH SIGNIFICANCE

Cutaneous melanoma is the result of malignant transformation of melanocytes that characterizes 1–2% of the total cell population found in the human epidermis. Since the mid-1960s melanoma represented considerable public health burden due to its substantial rise in incidence of about 3–8% per year in Caucasian populations worldwide (Thompson et al., 2005). Patients diagnosed with metastatic melanoma to distant sites have a median survival of only 4–6 months (Guida et al., 2012). Considering how sun and UV exposure are considered as critical environmental risk factor for melanoma, the effectiveness of DNA repair of UV-induced DNA lesions is directly responsible for cancer prevention and a list of DNA repair genes that are involved in developing distant metastasis or survival has been developed (Kauffmann et al., 2008). In this study, we focused on immune targeting of DNA-RP associated with resistance to CT and how HSP90i might beneficially modulate such recognition. In particular, while HSP90i are expected to decrease melanoma resistance to subsequent CT, we proposed that treated tumor cells will also become more susceptible to immune-mediated killing by DNA-RP-specific $CD8^+$ T cells, and that combination therapy integrating HSP90i and specific vaccination against HSP90 client DNA-RP will provide a more effective and durable treatment option for patients (in our case mice) with melanoma. In order to facilitate translation of our findings to the clinic, we used HSP90i that are currently in phase III clinical trials and took precautions to make sure that the lowest effective dosage of drugs was applied to avoid toxicity and to increase the treatment's safety index. ***Innovation*.** No studies have thus far focused on the ability of HSP90i to conditionally increase the degradation and subsequent presentation of DNA-RP-derived peptides in MHC class I complexes to the immune system *in vivo*. We hypothesized that HSP90i will upregulate the stoichiometry of a pool of HSP90 client protein-derived peptides presented in the

global “repertoire” of MHC class I/peptide complexes presented on the tumor cell surface, allowing for improved tumoricidal action of specific CD8^{+} T effector cells. If validated, these studies will serve as a foundation for the design and clinical implementation of novel combination immunotherapies for patients with melanoma.

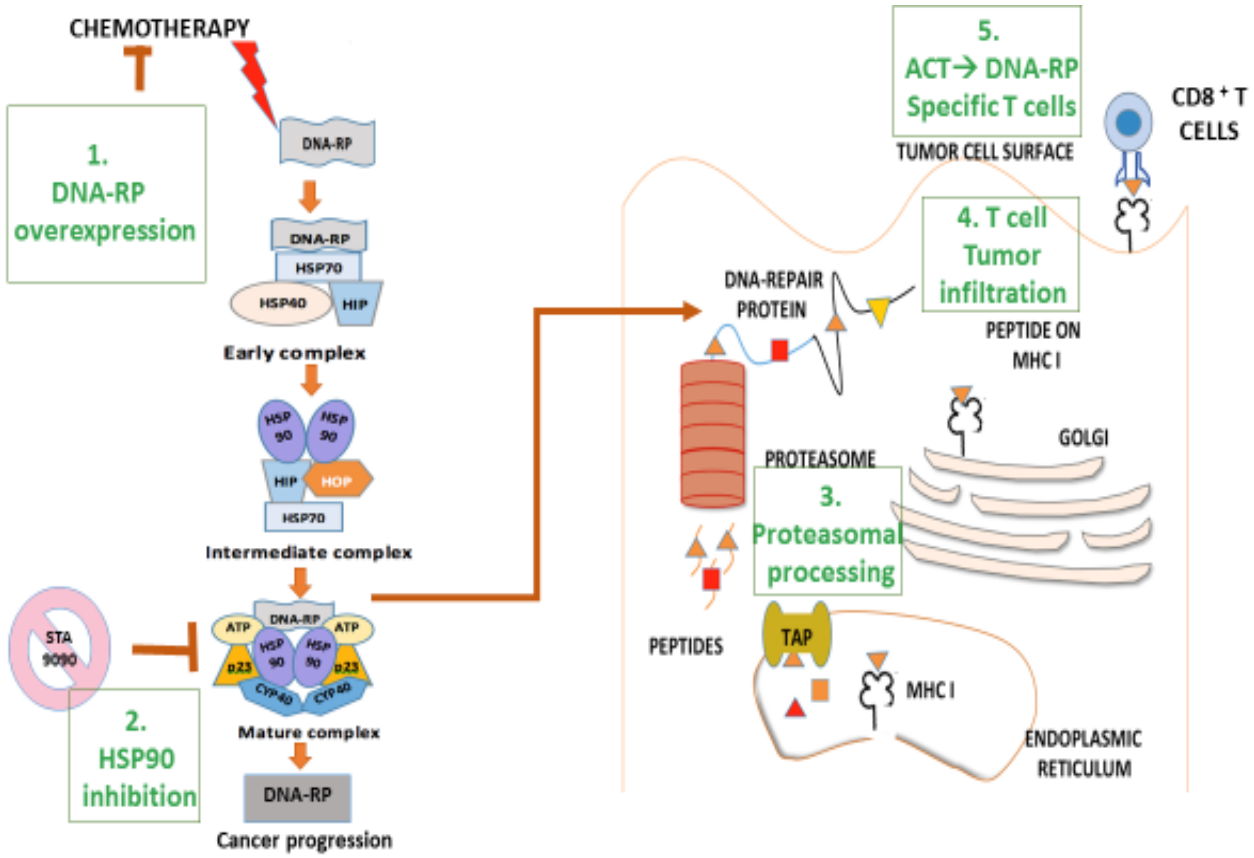


Figure 8. Thesis Rationale.

Chemotherapy causes many single strand and double strand lesions in cancer cells that induce corollary DNA repair mechanisms. In this process DNA-RPs become overexpressed in cancer cells, in association with resistance to genotoxic chemotherapies. Many DNA-RPs that play key roles in the DSB pathway are clients of HSP90, with HSP90 serving to maintain the structural and functional stability of these DNA-RP, leading to enhance tumor cell survival and the progression of chemotherapy-resistant disease. Our hypothesis posits that if we inhibit HSP90 using inhibitors like STA9090, these will destabilize the pool of tumor-sustaining DNA-RPs by forcing them to undergo proteasomal degradation. This provides a conditional pool of peptide substrates for loading into MHC class I complexes that may be recognized for several days by therapeutic anti-DNA-RP specific CD8^{+} T cells that may be

elicited via vaccination or delivered by ACT. By also conditioning the TME (by removing regulatory cells), HSP90i may allow therapeutic T cells to mediate their tumoricidal activity in an unopposed manner, resulting in improved treatment benefits.

The major hypothesis to be tested in this project is that HSP90i can conditionally sensitize melanoma cells to the tumoricidal action of CT as well as $CD8^+$ T cells reactive against overexpressed DNA-RP that constitute HSP90 client proteins. The Specific Aims of my thesis project are:

Specific Aim 1: To characterize the expression of DNA-RP client proteins by melanoma cells and determine the comparative anti-melanoma efficacy of combined CT +/- HSP90i therapy *in vitro* and *in vivo* (Chapter 3).

Specific Aim 2: To explore the anti-melanoma efficacy of combined therapy using HSP90i and specific vaccines or adoptive-transfer of $CD8^+$ T cells reactive against DNA-RP *in vivo* (Chapter 4).

2.0 MATERIALS AND METHODS

2.1 MICE

Six- to ten-week-old female C57BL/6 (H-2^b mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen-free animal facility in the South Biomedical Sciences Tower at the University of Pittsburgh. All animal experiments were performed in accordance with a University of Pittsburgh Institutional Animal Care and Use Committee (IACUC)-approved protocol.

2.2 TUMOR CELL LINES

The B16 murine melanoma (H-2^b) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). B16 is an HLA-$A2^{neg}$, mMART-1^+, $mgp100^+$ melanoma cell line (syngenic to the H-2^b background of C57BL/6 mice) The murine $BRAF^{V600E}PTEN^{-/-}$ melanoma cell line BP (Cooper et al., 2014) was kindly provided by Dr. Jennifer Wargo (University of Texas M.D. Anderson Cancer Center, Houston, TX) and then selected for resistance to the BRAF inhibitor, Dabrafenib (20 μM) for 1 month *in vitro*, yielding the BP20 cell line, which established and progressed more uniformly than the parental tumor cell line after s.c. implantation in C57BL/6 mice (Fecek et al., unpublished results). The human metastatic

melanoma cell lines, Mel526 (HLA-A2+) and Mel624 HLA-$A2^{+}$) were the kind gifts of Dr. Steven Rosenberg (National Institutes of Health, Bethesda, MD). The EL4 (H-2^{b}, ATCC) thymoma was used as a negative control target for $CD8^{+}$ T cell recognition assays. All tumor cell lines except EL4 were cultured in complete medium (CM1; RPMI 1640 media supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L L-glutamine, and 10% heat-inactivated fetal bovine serum; all purchased from Life Technologies, Grand Island, NY) in a humidified incubator at 37°C and 5% CO_2. EL4 cells were cultured in complete medium (CM2; DMEM medium supplemented with 100 μg/mL streptomycin, 10 mmol/L L-glutamine, and 10% heat-inactivated fetal bovine serum; all purchased from Life Technologies, Grand Island, NY) All cell lines were screened as negative for adventitious pathogens and for authentication by IDEXX BioResearch (Westbrook, ME).

2.3 WESTERN BLOTTING

Melanoma cell lines were grown to 80–90% confluence and then incubated with Temozolomide (50 μM, Selleck Chemicals, Houston, TX) for 12h, in the absence or presence of the HSP90i STA9090 (50 μM; Synta Pharmaceuticals, Lexington, MA) and/or the proteasome inhibitor MG132 (10 μM; Sigma-Aldrich, St. Louis, MO) for an additional 12h. In the test comparing DNA-RP expression with different chemo-toxic drugs +/- HSP90i, Dabrafenib (20 μM), Dasatinib (100 μM), Doxorubicin (0.1 μM), B16 murine melanoma cells were treated overnight for 12h. Alternatively, animals were euthanized, and progressively growing tumors from control or TMZ +/- HSP90i treated tumor-bearing mice were isolated by surgical dissection. Harvested cells/tissues were then incubated with lysis buffer. Lysates were cleared by centrifugation at

13,500 x g for 10 min, and proteins in the lysate resolved by SDS-PAGE prior to electro-transfer onto polyvinylidene difluoride (PVDF) membranes, as previously described (Kawabe et al., 2009). After blocking with 5% non-fat dry milk (NFDM) for 30 min, the membrane was incubated overnight with primary antibodies at 4^{o}C for specific anti-DNA repair proteins, HSP90 or β-actin. The primary antibodies used against DNA-RP were: Rabbit anti-ATR (1:15,000 dilution), mouse anti-PRKDC (2 µg/ml), mouse anti-MRE11 (3µg/ml), rabbit anti-NBN (1:10,000 dilution), rabbit anti-RAD50 (1:1000 dilution), mouse anti-RAD51 (1 µg/ml), rabbit anti-HSP90 (2 µg/ml), or rabbit β-actin pAb antibodies (1:10,000 dilution; all Abs from Abcam, Cambridge, UK). Blots were imaged for 1- 5 minutes on Kodak X-Omat Blue XB-1 film (Eastman Kodak, Rochester, NY) after incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig or goat anti-mouse Ig antibodies (1:10,000 dilution for both; Santa Cruz Biotechnology) and the Western Lighting chemiluminescence detection kit (Perkin-Elmer, Waltham, MA).

2.3.1 Densitometry analysis of protein bands

Densitometry analysis was performed using a GDS 8000 bioimaging system and LabWorks4.6 software (UVP, LLC, Upland, CA). Relative optical densities were calculated for specific protein of interest and loading control (β-actin) for all lanes using one of the lanes as a reference. Adjusted densities were then calculated for each sample by normalizing the relative density of the protein of interest to the loading control for the same.

$$\text{Adjusted density} = \frac{\text{Density of the protein of interest}}{\text{Density of the loading control}}$$

2.4 QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (QPCR).

Total RNA was extracted from either treated melanoma cell lines or total resected tumors from treated mice using the RNeasy Plus Mini Kit (Qiagen) including the gDNA Eliminator column. The Nanodrop ND-1000 (CellBio SpA, Milan, Italy) was used to check the quality and quantity of RNA. Total RNA (1µg) was reversed transcribed into cDNA using the High Capacity RNA to cDNA kit (Life Technologies) and the cDNA added to Fast SYBR® Green Master Mix (Life Technologies) and used for quantitative PCR. Reactions were performed on a StepOnePlus™ Real-Time PCR thermocycler (Applied Biosystems) using the recommended cycling conditions. All mRNA expression levels were normalized to the expression of the cellular housekeeping gene product Hprt. Primer sequences were selected using Primer-BLAST Genbank and are listed in Table 3.

Table 3. Real-time (qPCR) Primers used to Amplify Murine DNA-RP Transcripts in this study.

DNA-RP	Primer (forward 5'-3')	Primer (reverse)	Amplicon size (bp)
Atr	TTGGAAGGGCAGCAAAAGGA	CTCCAGAGACGGATGCAGAC	90
Prkdc	GACAAGTGCAGAAATGGAAGCA	CAGCCTGGCTTCAGAAGAGT	88
Mre11	GCCCCACAGATCCACTTGAC	TTCCTCTAACTGCATCTTTCTCCA	96
Nbn	CGGCTCCAGGAGAACCATAC	ATGCCACAGTTTTTCCTCCCA	70
Rad50	GCGTGCGAAGTTTTGGGATA	AATGATGGTCGTCTTCCCCG	107
Rad51	GCTGATGAGTTTGGTGTCGC	TTGGGATCTGCAGCGAACAT	86

2.5 ADOPTIVE CELL THERAPY

2.5.1 Selection of MHC class I-presented DNA-RP peptide epitopes

Specific DNA-RP peptides used in this study are listed in Table 4. These peptides were selected based on ranked scores generated in a web-based algorithm. Used peptide binding algorithms (SYFPEITHI, BIMAS and IEDAR) predictive of the ability of peptides to be produced from parental proteins by proteasomal processing combined with their ability to bind (and be presented by) either H-2D^b or H-2K^b class I molecules (http://www.iedb.org/). All 30 peptides were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry by the University of Pittsburgh Cancer Institute Peptide Synthesis Facility (a Shared Resource). Peptides were >96% pure based on high performance liquid chromatography profile and mass spectrometric analysis performed by the University of Pittsburgh Cancer Institute Protein Sequencing Facility (a Shared Resource).

Table 4. Mouse DNA-RP Peptides Sequenced.

DNA-RP	Peptide Sequence	Length (H-2 restrict)	Prediction Score
ATM	SSFPFMLL SSYKFLPL FMLLNYTSI	8 (K^b) 8 (K^b) 9 (D^b)	0.10 0.10 0.10
ATR	LSFAYGLL MNVQNVEFI	8 (K^b) 9 (D^b)	0.10 0.20
BRCA1	KSCMNQVTV AQRFFHQL	9 (D^b) 8 (D^b)	0.40 1.00
BRCA2	RALNNYRQM IDIVNTLPL	9 (D^b) 9 (D^b)	0.10 0.20
CHEK1	HMLVNSQLL SNLDFSPV MTRFFTKL	9 (D^b) 8 (K^b) 8 (K^b)	0.10 0.25 0.30
CHEK2	KLYFYQML GTFVNTELI	8 (K^b) 9(D^b)	0.25 0.40

Table 4 (continued)			
PRKDC	LFYKFVPL SALINLVEF	8 (K^b) 9 (D^b)	0.10 0.10
ERCC1	LSLRYHNL	8 (K^b)	0.10
XRCC6	SSTLFSAL QHFRNLEAL	8 (K^b) 9 (D^b)	0.10 0.10
MRE11	NSWFNLFVI VNYQDGNL	9 (D^b) 8 (K^b)	0.10 0.20
NBN	SAPVNMTTY VGITNTQLI	9 (D^b) 9 (D^b)	0.10 0.20
RAD50	RQIKNFHEL RNYNYRVV	9 (D^b) 8 (K^b)	0.10 0.20
RAD51	KELINIKGI VESRYALL	9 (D^b) 8 (K^b)	0.70 0.75
XRCC1	RQYMFSSL NPVENFRFL	8 (K^b) 9 (D^b)	0.10 0.20

2.5.2 *In vitro* generation of bone marrow–derived dendritic cells

Dendritic cells were generated from bone marrow precursors isolated from the tibias/femurs of C57BL/6 mice. Bone marrow cells were cultured in CM1 supplemented with 10% heat-inactivated fetal bovine serum, 1,000 units/mL recombinant murine granulocyte/macrophage colony-stimulating factor (rmGM-CSF) and 1,000 units/mL rmIL-4 (Peprotech) at 37°C in a humidified, 5% CO_2 incubator for up to 5-6 days.

2.5.3 Adenoviral infection of dendritic cells

Five million (day 5 cultured) dendritic cells were infected at a multiplicity of infection of 50 with recombinant adenoviruses encoding mouse IL-12p70 (Ad.IL-12) (i.e., DC.IL12; known to

promote T helper-independent activation of Type-1 $CD8^+$ T (Tc1) cells) as previously described (Zhao et al., 2011). After 48 h, infected dendritic cells (i.e., DC.IL12) were harvested. They were pulsed for 4h at 37ºC with pools of peptides derived from DNA-RP as indicated (with each individual peptide present at a concentration of 1 μM). **Viral vectors.** The Ad.mIL-12p70 recombinant adenoviral vectors were produced and provided by the University of Pittsburgh Cancer Institute Vector Core Facility.

2.5.4 Generation of DNA-RP-specific $CD8^+$ T cells for adoptive transfer therapy

To generate Ag-specific $CD8^+$ T cells for adoptive cell transfer experiments, naïve C57BL/6 (tumor-free) mice were vaccinated s.c. (right flank) on days 0, 7 and 14 with a vaccine consisting of syngenic bone marrow-derived DC.IL12 that were pulsed for 4h at 37ºC with pools of peptides derived from DNA-RP as indicated. One week after the 3rd vaccination (i.e., day 21), the animals were euthanized, spleens harvested and $CD8^+$ splenic T cells ($MACS^{TM}$-selected; Miltenyi Biotec, Auburn, CA). T cell specificity was determined in IFN-γ ELISA against melanoma vs. control EL4 vs. DNA-RP peptide-pulsed EL4 target cells. For adoptive cell therapy experiments, 1-5 million $CD8^+$ splenic T cells were fluorescently labeled using the cell tracker Deep Red Dye (5 μM, Thermofisher) for 30 min at 37ºC, just prior to washing and i.v. injection (in PBS) into melanoma-bearing mice.

Figure 9. Adoptive Cell Therapy in Melanoma-bearing Mice.

Naïve C5BL/6 mice were vaccinated thrice with DC.Ad.IL12 pulsed with DNA-RPs, 1 week after the 3rd vaccination $CD8^+$ splenic T cells were obtained and fluorescently labeled using the cell tracker deep red dye before intravenous (i.v.) injection into the tail vein of B16 melanoma-bearing mice.

2.6 TUMOR ESTABLISHMENT AND THERAPY MODELS

Tumors were established by injection of 1 x 10^5 B16 melanoma cells sub-cutaneously (s.c.) into the right flank of syngeneic C57BL/6 mice. For therapy models, tumors were allowed to establish through day 6, at which time animals were randomized into cohorts of 5 mice/group, with each cohort exhibiting a mean tumor size of approximately 35 mm^2. For standard chemotherapy experiments, mice were treated with intraperitoneal (i.p.) injection of TMZ (50 mg/kg/day) in 100 µl DMSO (Sigma-Aldrich, St. Louis, MO) or vehicle control (DMSO) on days 6 and 7. For combinational chemoimmunotherapy treatment, tumor-bearing mice were treated with TMZ (i.p., 50 mg/kg/day in 100 µl DMSO) or vehicle control (DMSO) on days 6 and 7, STA9090 (i.p., 25 mg/kg/day in 100 µl DMSO) or vehicle control (DMSO) on days 8 and

15, and adoptive transfer of 1-5 x 10^6 anti-DNA-RP $CD8^+$ T cells (i.v. in the tail vein, in PBS) or vehicle control (PBS) on days 9 and 16. Tumor size (in mm^2) was determined as the product of orthogonal caliper measurements monitored every 3 to 4 days thereafter. Mice were euthanized when tumors became ulcerated or they reached a size of 400 mm^2, in accordance with IACUC guidelines.

2.7 IFN-γ ELISA

For tumor recognition assays, splenic $CD8^+$ T cells were co-cultured with melanoma cells, negative control EL4 (H-2^b; thymoma) or EL4 cells pre-pulsed (for 4h at 37^{o}C) with DNA-RP-derived peptides (1 μM each) for 48h, after which, cell-free supernatants were harvested and assessed for mIFN-γ concentration using a specific ELISA (BD Biosciences). Data are reported as mean ± SD of quadruplicate determinations.

For tumor recognition assays, enriched splenic $CD8^+$ T cells (i.e., single-cell suspensions of splenocytes were first obtained from mechanically-disrupted spleen and then $CD8^+$ T cells were negatively selected) from vaccinated and naïve mice were co-cultured with freshly-irradiated (100 Gy at room temperature from a ^{137}Cs irradiator (Gammacell40, Atomic Energy of Canada Limited, Mississauga, Ontario, Canada at a dose rate of 0.87 Gy/min) B16 tumor cells that had been previously treated with STA9090 (0.1 nM for 12 h) with or without TMZ (50μM for 12 h), after which, cell-free supernatants were harvested and assessed for mIFN-γ concentrations using a specific ELISA (BD Biosciences). The data are reported as mean ± SD of triplicate determinations.

2.8 IMMUNOFLUORESCENCE STAINING AND IMAGING

Tumor tissue samples were embedded in ornithine carbamyl transferase medium (Tissue-Tek; Sakura Finetek U.S.A., Inc., Torrance, CA), frozen, and stored at −80°C. Five-micrometer tissue sections were prepared using a cryostat microtome, mounted on Superfrost Plus (Fisher Scientific, Pittsburgh, PA) slides, and stored at −80°C, followed by immunofluorescence staining and microscopy. The following primary antibodies were used for staining sections: rat anti-mouse CD3, rabbit anti-mouse T-bet (Santa Cruz Biotechnology, San Diego, CA). Secondary antibodies included: Cy3-conjugated goat anti-rat Ig and Alexa488-conjugated goat anti-rabbit Ig (both from Jackson ImmunoResearch, West Grove, PA). TUNEL staining for detection of apoptotic cells was performed using a cell death detection kit (Roche Diagnostics, Indianapolis, IN) per the manufacturer's instructions. All tissue sections were then briefly incubated with the nuclear counterstain 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). After washing, sections were then covered in Gelvatol (Monsanto, St. Louis, MO) and a coverslip applied. Slide images were acquired using an Olympus Provis microscope (Olympus America, Center Valley, PA). Isotype control and specific antibody images were taken using the same level of exposure on the channel settings. Metamorph (Molecular Devices, Sunnyvale, CA) software was used for labeled cell quantification.

2.9 FLOW CYTOMETRY

Single-cell suspensions were directly stained with APC-conjugated anti-CD8 (eBioscience), Deep-red dye cell tracker (Thermofisher) and Annexin V-FITC from the apoptosis detection kit

Staining kit (Abcam) according to the manufacturer's instructions. Fluorescently-stained cells were assessed using an LSR II flow cytometer (Beckman Coulter), with data analyzed using FlowJo software (Tree Star, Inc.).

2.9.1 Cell cycle analysis by flow cytometry

Cells were seeded and allowed to attach overnight. Following the relevant treatments Temozolomide +/- STA9090, floating and adherent cells were collected at the end of 24 h, washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol. The cells were then treated with 80 µg/ml RNase A and 50 µg/ml propidium iodide (Invitrogen-Molecular Probes, Carlsbad, CA) for 45 min at 37^{o}C. The stained cells were analyzed using a LSR II flow cytometer (Beckman Coulter), with data analyzed using FlowJo software (Tree Star, Inc.).

2.10 STATISTICAL ANALYSES

Comparisons between groups were performed using a two-tailed Student's t test or area under the curve or one-way Analysis of Variance (ANOVA) with post-hoc analysis, as indicated. All data were analyzed using SigmaStat software (Systat Software, USA). Differences with a p-value < 0.05 were considered as significant.

3.0 TARGETING HSP90 TO COMBAT DRUG-RESISTANT CANCER

3.1 INTRODUCTION

Cancer is the second most common cause of death worldwide. From the latest statistics released by the International Agency for Research on Cancer (IARC), the global burden of cancer has increased to an estimate of 14 million new cases per year, with cancer deaths estimated at up to 13 million annually. Developing countries are affected disproportionately, with more than 60% of new cases and 70% of cancer related deaths occurring in Africa, Asia, Central and South America (Globocan 2012, IARC). This situation is made worse in developing nations due to poor early detection of disease and the lack of access to first-line, “standard of care” treatments including chemotherapy, radiotherapy, targeted immunotherapy and hormone therapy. Considering the heterogeneous nature of cancer cells in a given tumor lesion, it is not at all surprising that these cell populations rapidly develop compensatory resistance mechanisms to circumvent any given specific form of treatment. In order to combat such resistance, many studies have considered combining existing, conventional treatments with HSP90i in order to destabilize a broad range of client proteins that underlie the acquired drug-resistant phenotype of cancer cells.

3.2 TARGETING HSP90 IN CANCER THERAPY

Heat shock protein 90 (HSP90), an ATP-dependent molecular chaperone accounts for about 1-2% of total protein under normal conditions, but this level increases to 4-6% under stress conditions (Hickey et al., 1986). On the other hand, in cancer cells, HSP90 is frequently overexpressed and plays an important role in the maintenance of protein conformational integrity, stabilization of a number of oncogenic/survival proteins, providing a cytoprotective response to hypoxic and acidic microenvironments and preventing their proteasome-mediated degradation, thereby supporting tumor progression and metastasis (Whitesell & Lindquist, 2005).

Most chemotherapeutic agents target a limited number of individual proteins or signaling mechanisms, which might lead to the compensatory induction of alternate pro-tumor biologic pathways. Use of HSP90i would theoretically lead to a coordinate disruption of multiple compensatory pathways that support cancer cell survival and metastasis (Koga et al., 2009). Additionally, since HSP90 is highly overexpressed in tumors, this makes HSP90i-based intervention somewhat more selective in its site of action (Kamal et al., 2003). As a consequence, the use of HSP90i has gained substantial inertia for consideration as a novel cancer therapeutic strategy to combat disease resistance that is commonly developed in response to conventional cancer treatment modalities.

3.2.1 Fighting drug-resistance with HSP90i

To date, chemotherapy and radiotherapy remain the most commonly applied treatments for cancer, but these both evoke the development of a high frequency of treatment-resistant disease. In order to combat this resistance HSP90 inhibitors are being used in combination with standard

of care regimens. The HSP90i, 17-AAG and Geldanamycin have been used in several studies to fight developed resistance against commonly-used chemotherapy drugs, such as cisplatin and paclitaxel. In almost all cases, the synergistic anti-cancer activity of the drugs has been observed (Lu et al., 2012; Solit et al., 2003; Tatokoro et al., 2012; Vasilevskaya et al., 2004). In an *in vivo* study of combination of 17-AAG with cisplatin showed that 17-AAG sensitized nude mice to the anti-tumor action of cisplatin treatment (Ohba et al., 2010). After treatment with radiotherapy, DNA repair proteins (many of which represent HSP90 clients) become overexpressed, which may explain the enhanced therapeutic benefits of combining HSP90i with radiotherapy, leading to increased tumor cell death *in vitro* and *in vivo* (Schmidt-Ullrich et al., 2003; Dote et al., 2006; Jewell et al., 2010; Ha et al., 2011; Stecklein et al., 2012; Guida et al., 2012; Ko et al., 2012; Che et al., 2013; Acquaviva et al., 2014; Pennisi et al., 2015).

Temozolomide (TMZ, aka Temodar) is an alkylating drug. It has been FDA-approved for adult patients with refractory anaplastic astrocytoma since 1999 and for adult patients with glioblastoma multiforme (GBM) since 2005 ("FDA Approval for Temozolomide - National Cancer Institute," 1999). It is also used to treat melanoma patients. The resistance to TMZ has been correlated with overexpression of DNA repair proteins and/or DNA repair pathway deficiency (Zhang et al., 2012). Some studies with the older 1st generation HSP90 inhibitor, 17-AAG to fight the resistance against TMZ in human glioma cells did not induce synergy (Sauvageot et al., 2008; Ohba et al., 2010). However, a study using 17-DMAG on malignant glioma cells increased the cytotoxic effect of radiation and TMZ (Choi et al., 2014).

Even targeted cancer therapies can lead to the development of treatment-resistant disease. The anti-tumor activity of Vemurafenib, which inhibits signaling mediated by mutant oncogenic BRAF, was improved when combined with the HSP90i such as STA9090 and XL888

(Acquaviva et al., 2014). Similar results have been observed for therapeutic combinations that incorporate HSP90i (NVP-AUY922, IPI-504, 17-AAG) with anti-HER2 antibodies (Trastuzumab) in the setting of human breast cancer (Modi et al., 2011; Scaltriti et al., 2011; Wainberg et al., 2013), and HSP90i with imatinib (a KIT inhibitor) in the setting of gastrointestinal stromal cancers (Bauer et al., 2006; Smyth et al., 2012). In addition, ongoing studies have also effectively applied HSP90i to increase the sensitivity of breast cancer cells to hormone co-therapy.

Since HSP90i exhibit a degree of tumor selectivity (i.e., accumulation and retention in the TME), they should be applied at lower than maximal tolerated doses in combination protocols to limit off-target toxicities and to maximize on-target benefits to patients with cancer (Trepel et al., 2010; Neckers & Workman, 2012). HSP90i represent promising agents for use in combating drug-resistant cancers, preferably when used in combination approaches.

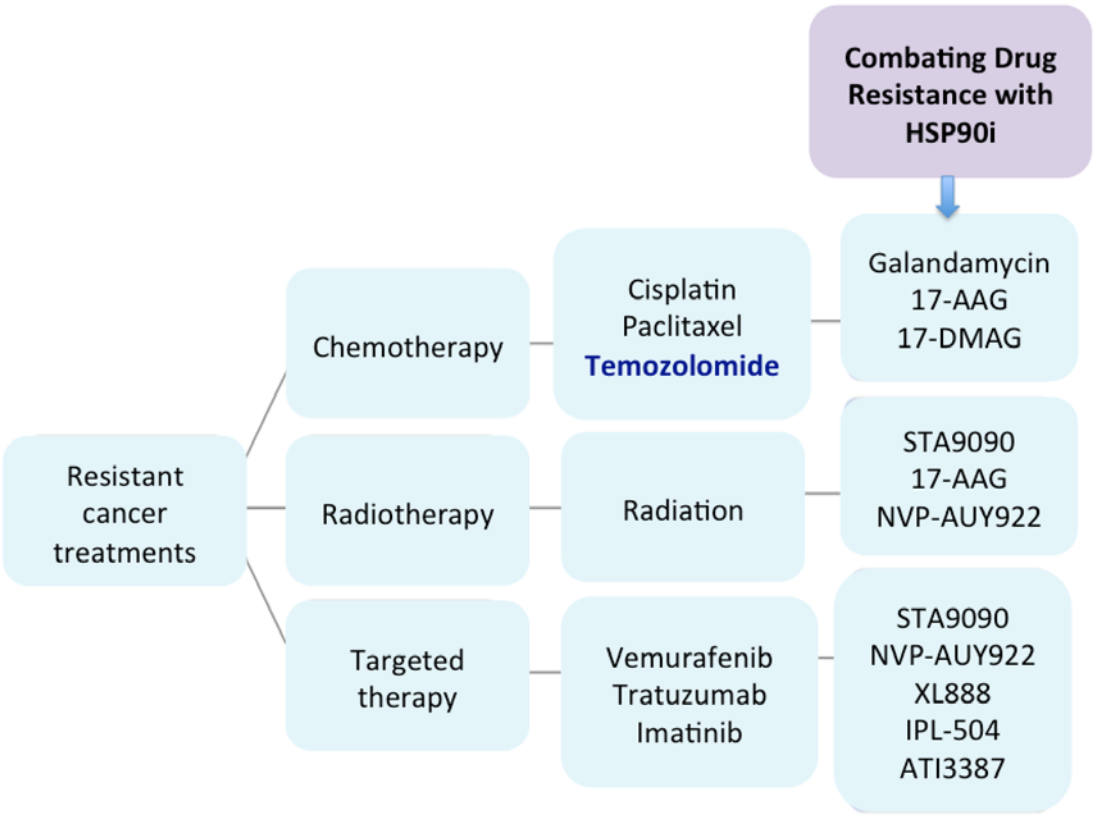


Figure 10. Combating Drug-resistant Cancer with HSP90 Inhibitors.

Combination with HSP90 inhibitors like Galandamycin, 17-AAG, 17-DMAG, STA9090 increases the sensitivity to otherwise resistant chemotherapy, radiotherapy or targeted therapy treatment modalities in cancer patients. Therapeutic targeting of HSP90 is predicted to block the pro-tumor impact of multiple oncogenic pathways associated with drug-resistant cancer progression and dissemination.

3.3 RESULTS

3.3.1 Melanoma develops resistance to the chemo-toxic drug Temozolomide (TMZ)

To check the efficacy of TMZ treatment, we looked at the effect of this drug on wild-type B16 melanoma cells. Tumor cells were pre-treated with 50 μM for 14 days *in vitro*, or established

B16 tumors were biopsied from the C57BL/6 mice after two daily doses of TMZ (50mg/kg/mouse/day injected i.p.). All cell lines were treated with increasing doses of TMZ overnight for 12h starting from 1 μM, 10 μM, 100 μM and 1000 μM. To analyze TMZ-induced melanoma apoptosis, tumor cells were stained using an Annexin-V FITC kit and analyzed by flow cytometry (Table 5). Results suggest a similar pattern of apoptotic sensitivity amongst melanoma cells treated with any dose of TMZ *in vitro* or *in vivo,* supporting the conclusion that melanoma cells are inherently resistant to TMZ treatment.

Table 5. B16 Melanoma Develops Resistance to TMZ.

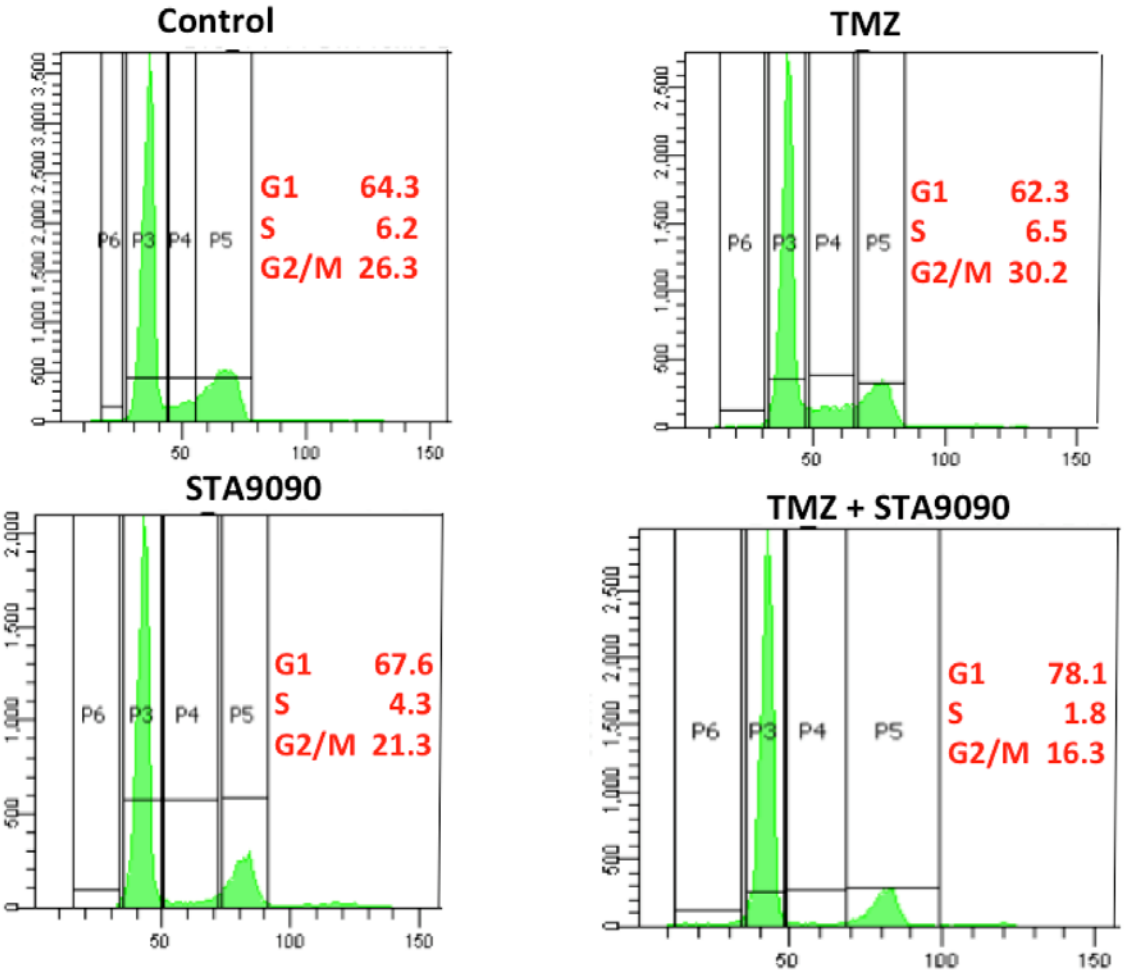
(A) **TMZ dose**	**(B)** **% of Total Apoptosis in B16 tumor cells TMZ treated** (*in vivo)*	**(C)** **% of Total Apoptosis in B16 TMZ pre-treated** (*in vitro)*	**(D)** **% of Total Apoptosis in B16** *(in vitro)*
UT	**1.9**	**1.6**	**2.1**
1 μM	**3.3**	**1.3**	**5**
10 μM	**5.2**	**3.5**	**3.3**
100 μM	**3.8**	**1.9**	**3.9**
1000 μM	**7.6**	**2.7**	**7.9**

In, (B) Wild-type B16 melanoma cells, (C) B16 cells pre-treated with 50 μM for 14 days in vitro, (D) established B16 tumors were biopsied from the C57BL/6 mice after two daily doses of TMZ (50mg/kg/mouse/day injected intra-peritoneally). All cell lines were treated with increasing doses of TMZ overnight for 12h starting from 1 μM, 10 μM, 100 μM and 1000 μM (A). To analyze TMZ-induced melanoma apoptosis, tumor cells were harvested and stained with PI + RNase for 45 minutes at 37oC, or stained with Annexin-V FITC and PI for 5 minutes at RT, prior to analysis using flow cytometry. The values in panels B, C and D represent the cumulative percentage of both late apoptotic cells and early apoptotic cells.

3.3.2 Sensitizing tumors to CT using HSP90i

In comparison with the treatment groups using TMZ alone, the combination of TMZ with STA9090 resulted in a >50-fold increase in tumor cell apoptosis. Hence, the combination therapy including HSP90i STA9090 appears to fight intrinsic/acquired tumor cell resistance to TMZ.

A



B

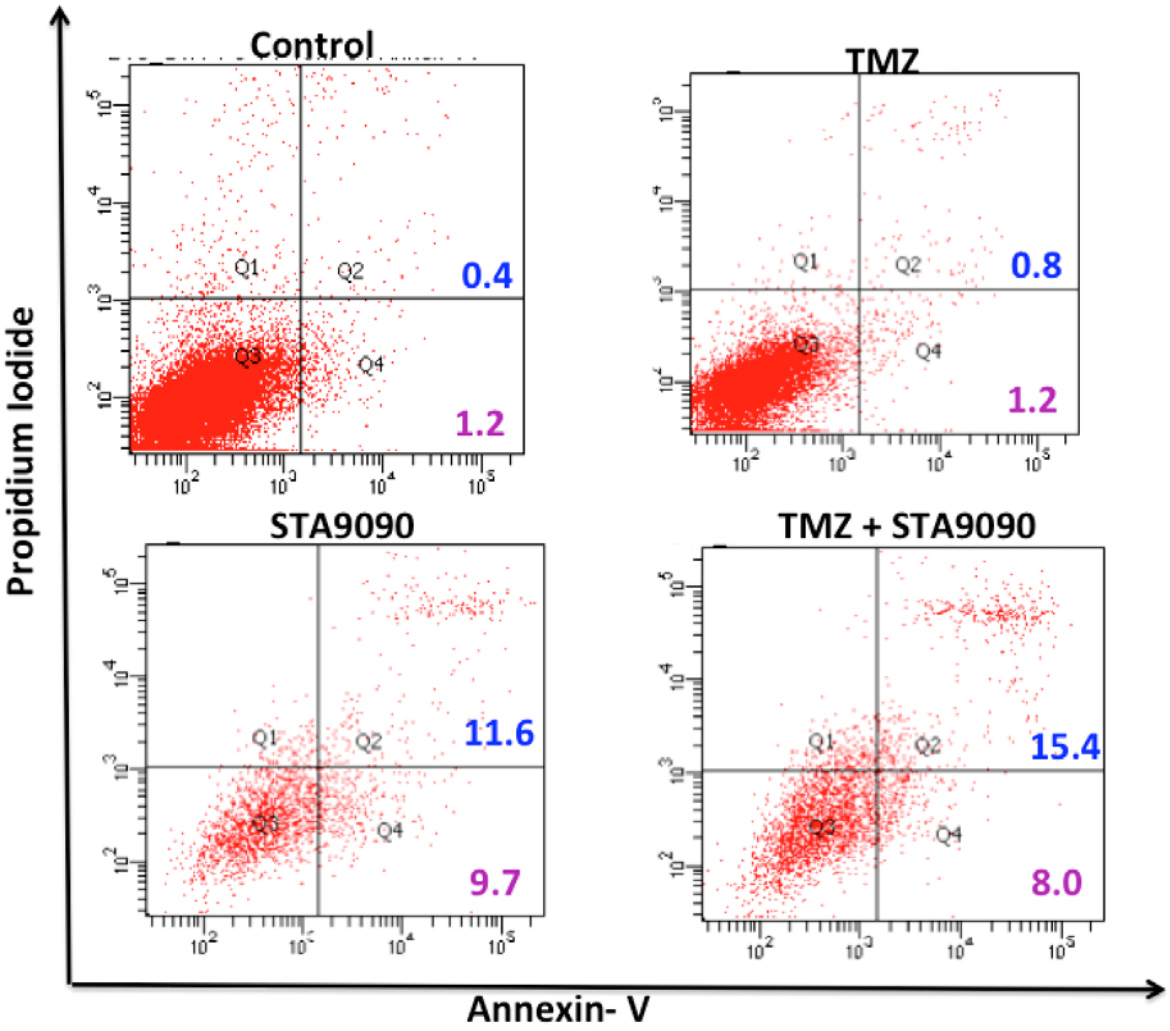


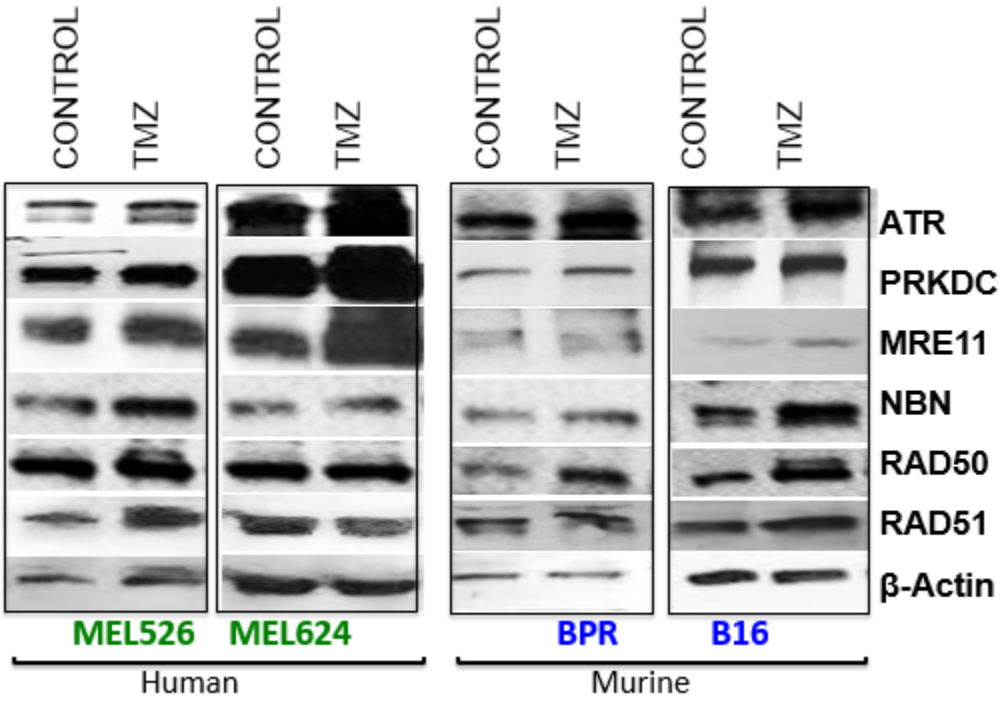
Figure 11. Treatment of B16 Melanoma Cells with Combined TMZ + HSP90i Results in Superior Blockade of G1 to S/G2/M Cell Cycle Transition and Enhanced Cellular Apoptosis *In Vitro.*

B16 melanoma cells were left untreated, or they were treated with TMZ (50 μM for 3h) +/- HSP90i STA9090 (25 μM for 16h) at 37°C. Cells were harvested and were fixed with 70% Ethanol overnight at 4°C and then (**A**) stained with PI + RNase for 45 minutes at 37°C, or (**B**) stained with Annexin-V FITC and PI for 5 minutes at RT, prior to analysis using flow cytometry. Q2 represents late apoptotic cells, Q3 represents viable cells and Q4 represents early apoptotic cells.

3.3.3 CT sensitization with HSP90i leads to loss of the S and G2/M checkpoints and increasing apoptotic tumor events

Preliminary studies were conducted on the B16 murine melanoma cell line treated with Temozolomide (TMZ- 50 μM-3 h) in the absence or presence of HSP90i –STA9090 (25 μM -16 h at 37ºC). Cells were harvested and fixed with 70% ethanol overnight at 4ºC and then stained with PI + RNase (Abcam) for 45 minutes at 37ºC prior to analysis using flow cytometry. The results suggest that melanoma treatment with the HSP90i STA9090 increases the tumor cell sensitivity to CT by limiting the transition from G1 to S and G2/M cell cycle checkpoint and by reversing the G2 arrest caused by TMZ (Figure 11).

A



B

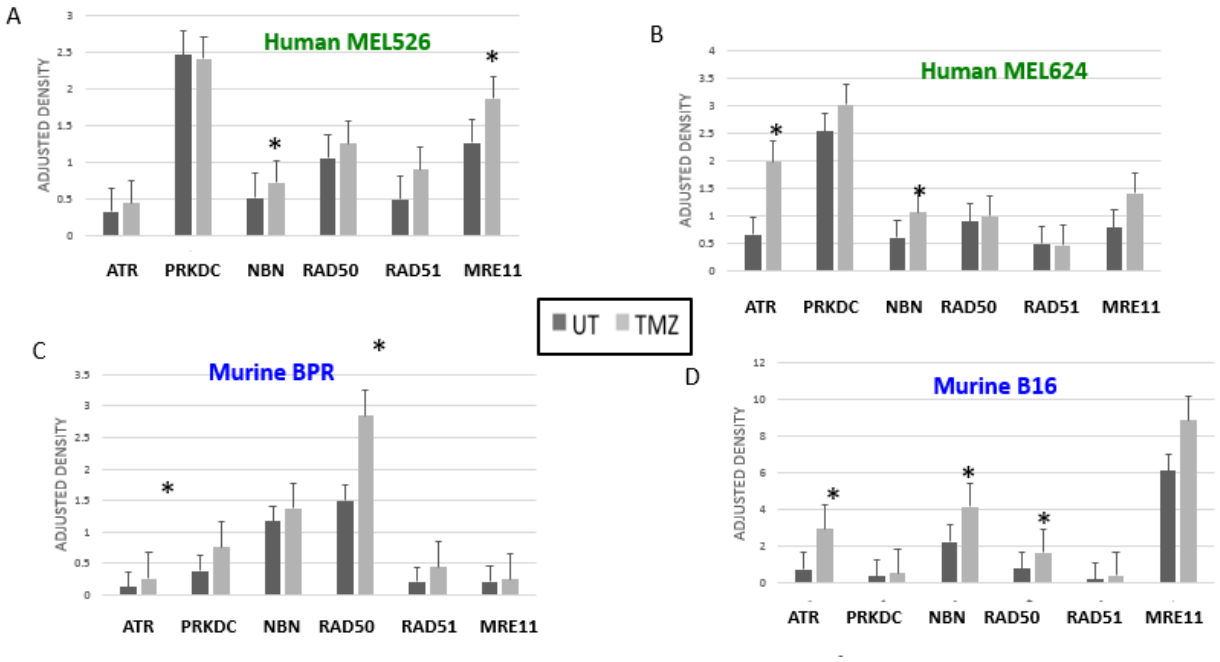
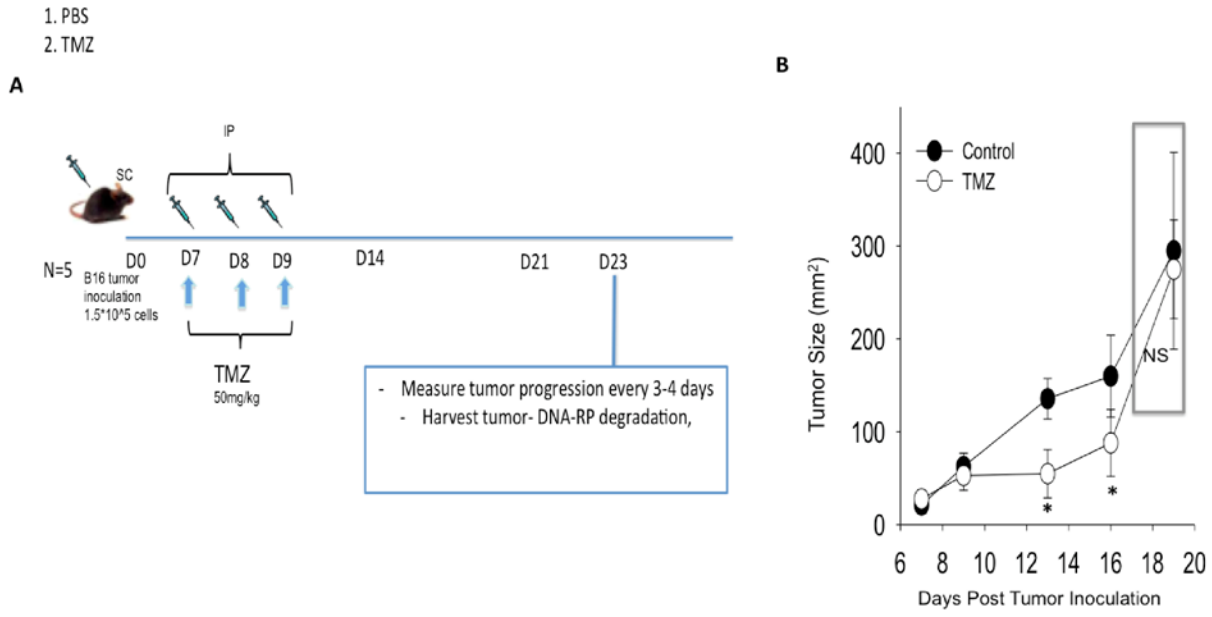


Figure 12. Treatment of Melanoma Cell Lines with TMZ Results in Upregulated Expression of a Subset of DNA-RP *In Vitro*.

In **A**, human (Mel526, Mel624) and murine (BPR20, B16) melanoma cell lines were cultured in the presence of TMZ (50 μM) for 12 h at 37ºC, at which time, tumor cells were lysed and SDS-PAGE resolved proteins analyzed by Western Blotting using specific antibodies reactive against the indicated DNA-RP or control β-actin (as described in the Materials and Methods). In **B (Ba-Bd)**. Specific DNA-RP protein bands in blots were quantitated by densitometry scanning and normalized to control β-actin as described in the Materials and Methods and all error bars reflect SEM, (Ba-MEL526, Bb- MEL624, Bc-BPR20, Bd- B16) with reported values reflective of three independent experiments performed in each instance, *, $p<0.05$.

3.3.4 TMZ promotes upregulated expression of a subset of DNA-RP in murine and human melanoma cell lines *in vitro*

To initially determine whether the genotoxic chemotherapeutic agent, TMZ could alter tumor cell expression of DNA-RP, melanoma cell lines were incubated *in vitro* with TMZ for 48h. Using Western Blotting analyses, we observed that TMZ treatment resulted in the coordinate increased expression of known DNA-RP (including ATR, PRKDC, MRE11, NBN, RAD50, RAD51) when compared with expression of the β-actin control protein by both murine (B16, BPR20) and human (Mel526, Mel624) melanoma cell lines, with the only exceptions being for PRKDC in Mel526 cells and for RAD51 in Mel624 cells, which may reflect an expected heterogenenity in genotype/phenotype amongst metastatic melanomas (Figure 12).



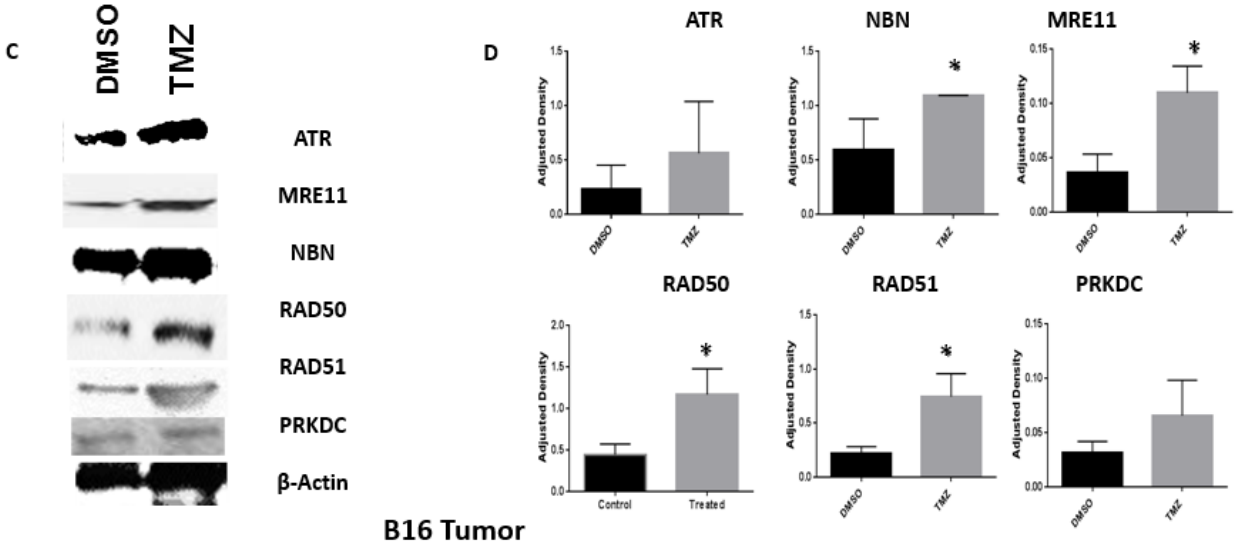


Figure 13. Melanoma Tumor Develops Resistance to TMZ and Results in Upregulated Expression of DNA-RP *in vivo*.

In **A**, C57BL/6 mice bearing established s.c. B16 melanomas (day 7) were treated i.p. with TMZ (50 mg/kg in DMSO on days 7-9) or with vehicle control. **B,** Tumor growth was monitored every 2-4 days through day 19, with mean tumor size (± SD) tumor size reported in mm^2. In **C,** melanoma isolated from the tumor of median size in each cohort on day 19 was solubilized and subjected to Western Blotting analysis. **D.** Specific DNA-RP protein bands in blots were quantitated by densitometry scanning and normalized to control β-actin as described in the Materials and Methods and all error bars reflect SEM. NS, not significant, *, $p<0.05$.

3.3.5 TMZ promotes upregulated expression of a subset of DNA-RP in murine and human melanoma cell lines *in vivo*

To determine whether similar results could be achieved *in vivo*, C57BL/6 mice were first implanted with B16 melanoma cells s.c. in the right flank, and tumors allowed to establish and progress for 6 days. On days 6 and 7, tumor-bearing mice were administered TMZ (50 mg/kg/day in DMSO) or vehicle control DMSO. As shown in **Fig. 13A**, TMZ-based therapy

slowed B16 melanoma growth for approximately one week, after which tumors developed drug-resistance and re-established control growth kinetics by day 19 of the experiment. Western Blotting analyses of day 19 harvested tumors revealed upregulated expression of DNA-RP in the TMZ-treated versus control cohorts (**Fig. 13C**).

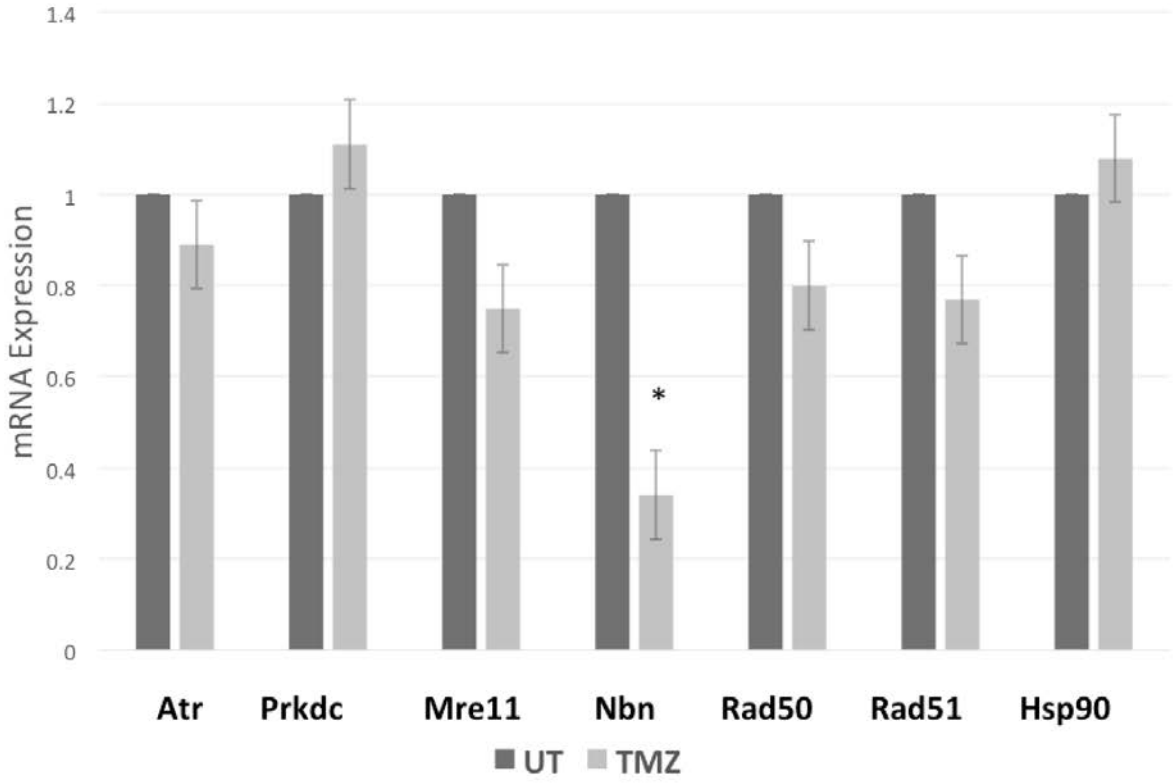


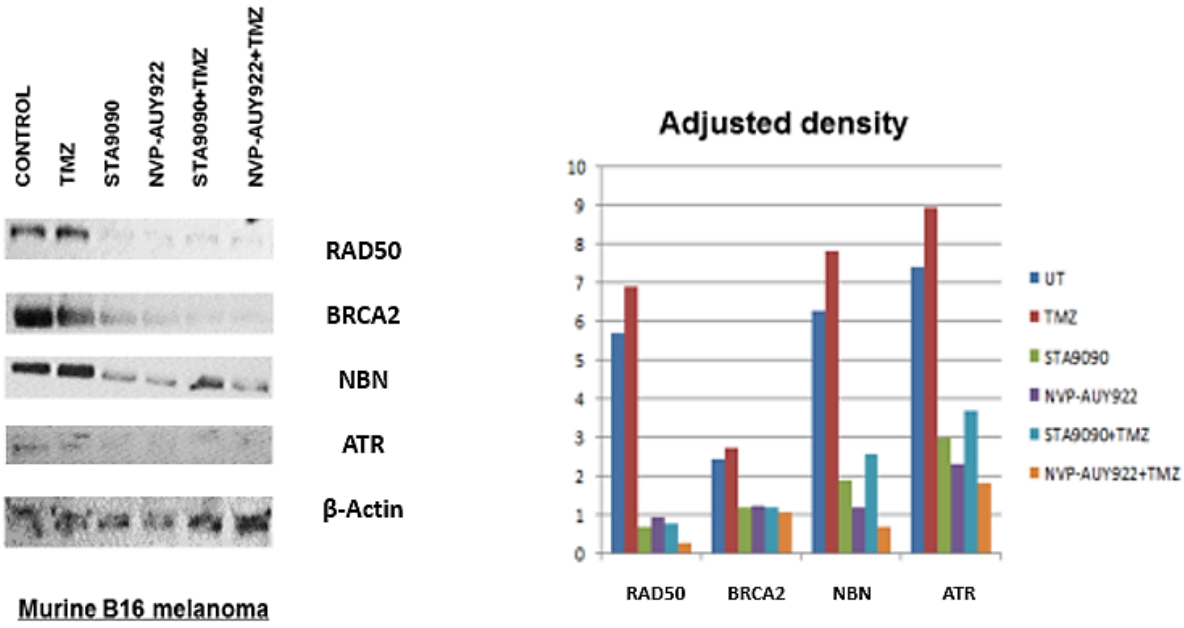
Figure 14. Effects of TMZ on the Transcription of Melanoma DNA-RP and HSP90.

The murine (B16) melanoma cell line was cultured in the presence of TMZ (50 μM) for 12 h at 37ºC. As described in the Materials and Methods, total RNA extracted was reverse transcribed to cDNA and used for qPCR. All mRNA expression levels were normalized to the expression of the cellular housekeeping gene product Hprt. Primer sequences were selected using Primer-BLAST Genbank and are listed in the Materials and Methods. All error bars reflect SEM, *, $p<0.05$.

3.3.6 Effects of TMZ on the transcription of melanoma DNA-RP and HSP90

Melanoma cells were treated with TMZ (50 μM for 12 h at 37^{o}C) *in vitro*, prior to cell harvest and extraction of cellular mRNA. Levels of DNA-RP transcripts were then quantitated using real-time qPCR (**Fig. 14**). With the exception of PRKDC, melanoma transcript levels for DNA-RPs were not significantly increased in response to treatment with TMZ. However, we did detect an increase in HSP90 mRNA expression after treatment with TMZ, which would support the hypothesis that TMZ-associated increases in melanoma expression of DNA-RP may be due to post-translational stabilization of these client proteins by TMZ upregulation of melanoma expressed HSP90.

A



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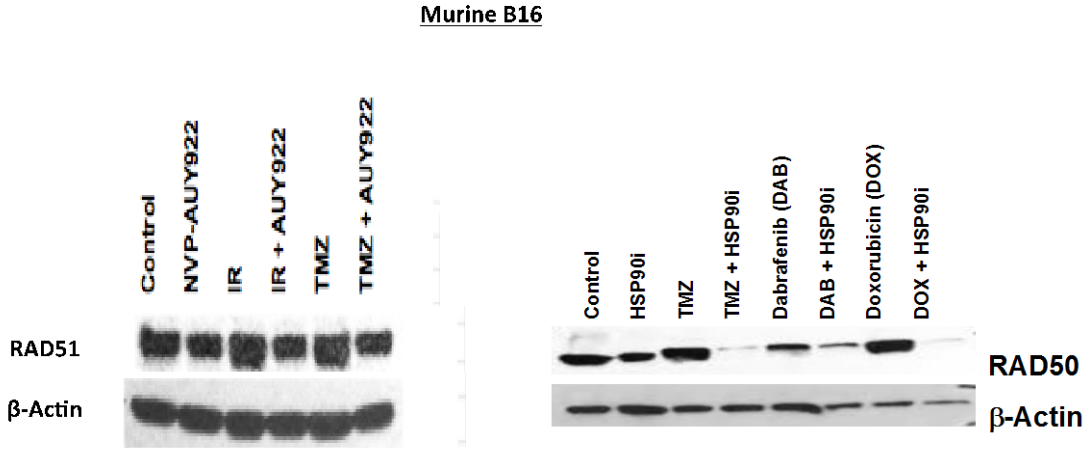


Figure 15. Genotoxic Chemotherapy agents, but Not BRAFi Dabrafenib, and Radiation Promote Increased Expression of DNA-RP RAD50 in B16 Melanoma cells that is Induced to Undergo Degradation by HSP90i *in Vitro*.

A, The B16 murine melanoma cell line was incubated in the absence or presence of TMZ (50 μM), +/- STA9090 (25 μM) for 12h or NVP-AUY922 at 37ºC, prior to cell solubilization and Western Blotting analysis for the DNA-

RP RAD50, BRCA2, NBN and ATR versus β-actin control proteins. Specific DNA-RP protein bands in blots were quantitated by densitometry scanning and normalized to control β-actin as described in the Materials and Methods. **B,** the B16 murine melanoma cell line was cultured in the absence or presence of TMZ (50 μM), IR 4 Gy , +/- NVP-AUY922 at 37ºC, prior to cell solubilization and Western Blotting analysis for the DNA-RP RAD51 versus β-actin control proteins. **C,** B16 cell line was incubated in the absence or presence of TMZ (50 μM), Dabrafenib (20 μM) or Doxorubicin (0.5 μM), +/- STA9090 (25 μM) for 12h at 37ºC, prior to cell solubilization and Western Blotting analysis for the murine DNA-RP RAD50 versus β-actin control proteins.

3.3.7 HSP90i promotes the degradation of DNA-RP

DNA-RPs that were upregulated as a result of TMZ treatment were degraded when treated in combination with the HSP90 inhibitors STA9090 and NVP-AUY922. A similar effect was observed with the other chemotoxic drug Doxorubicin and radiation as well. But, when treated with the BRAF inhibitor Dabrafenib, there was no overexpression of murine DNA-RP RAD50. However, when treated in combination with the HSP90i, STA9090, there was degradation.

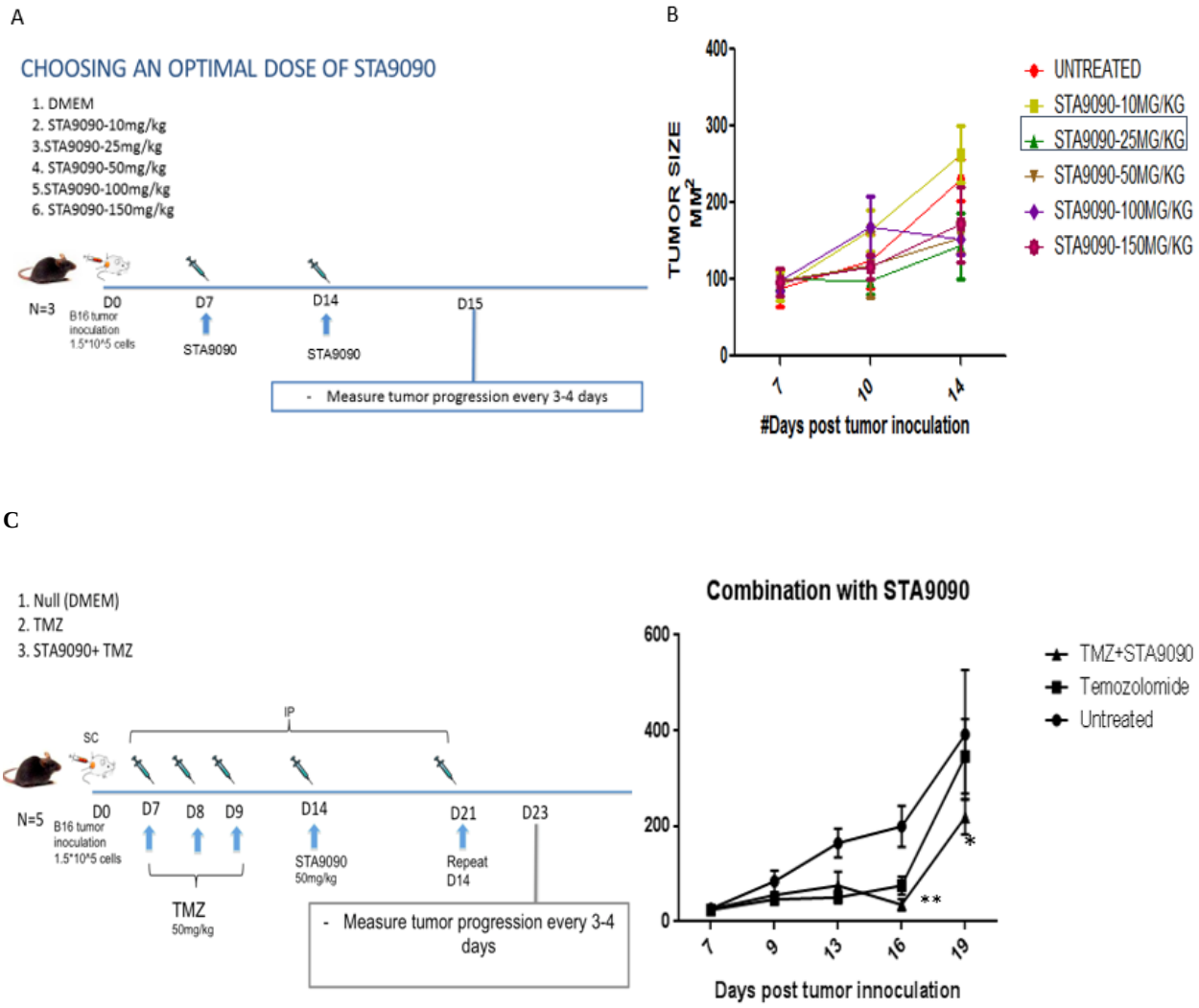


Figure 16. Optimizing the Dose of HSP90 for Combination Therapy of B16 Melanoma.

In **A**, C57BL/6 mice bearing established s.c. B16 melanomas (day 7) were treated i.p. with STA9090 (10, 25, 50, 100, 150 mg/kg in the vehicle as described on days 7-14) or with vehicle control. **B,** Tumor growth was monitored every 3-4 days through day 17, with mean tumor size (± SD) tumor size reported in mm^2. **C,** When STA9090 is used in combination with TMZ, it appears to provide superior anti-tumor efficacy based on inhibited tumor growth vs. control treatments **, $p<0.05$, * =$P < 0.1$ for combination.

3.3.8 Optimal HSP90i dose for combination to fight the TMZ resistance

To optimize combined therapy using TMZ + HSP90i STA9090, we first needed to establish an optimal dose of HSP90i monotherapy. In particular, we wanted to determine the lowest dose needed to achieve the greatest degree of therapeutic benefit. It is important to optimize the HSP90i dosing to achieve maximal anti-tumor benefits mediated by the immune response, as HSP90i can negatively impact DC and T cells when applied in high doses. DC expression of MHC class I molecules can also be reduced in the presence of high-dose (1 μM for 24-48h) HSP90i *in vitro* (Bae et al., 2013).

We initially chose five different doses of STA9090 10 mg/kg, 25 mg/kg, 50 mg/kg, 100 mg/kg and 150 mg/kg for analysis. C57BL/6 mice were first implanted with B16 melanoma cells s.c. in the right flank and tumors established to palpable lesions by 6 days. On days 7 and 14 (post-tumor inoculation), tumor-bearing mice were administered different doses of HSP90i STA9090 or vehicle control. Ultimately, we selected 25 mg/kg as an optimal dose for future experiments as it was the lowest dose that exhibited optimal efficacy against tumor progression versus TMZ monotherapy as shown in **Fig. 16**.

3.3.9 Identification of immunogenic peptides derived from DNA-RP for use in combination immunotherapies

To determine the impact of TMZ and/or HSP90i on the proteasome-dependent generation of MHC I-presented epitopes and consequent $CD8^+$ T cell recognition, we needed to first identify immunogenic peptide specific to DNA-RP.

For the purposes of investigating the immunogenicity of the 30 peptides described in Materials and Methods (**Table 4**), three groups of 10 pooled peptides were randomly generated and used to vaccinate naïve C57BL/6 mice thrice using a weekly schedule. One week after the final vaccination, $CD8^+$ splenic T cells cultured with EL4 cells that had been pre-loaded *in vitro* with individual DNA-RP-derived peptides (1 mM) after which, cell-free supernatants were harvested and assessed for mIFN-γ concentration using ELISA.

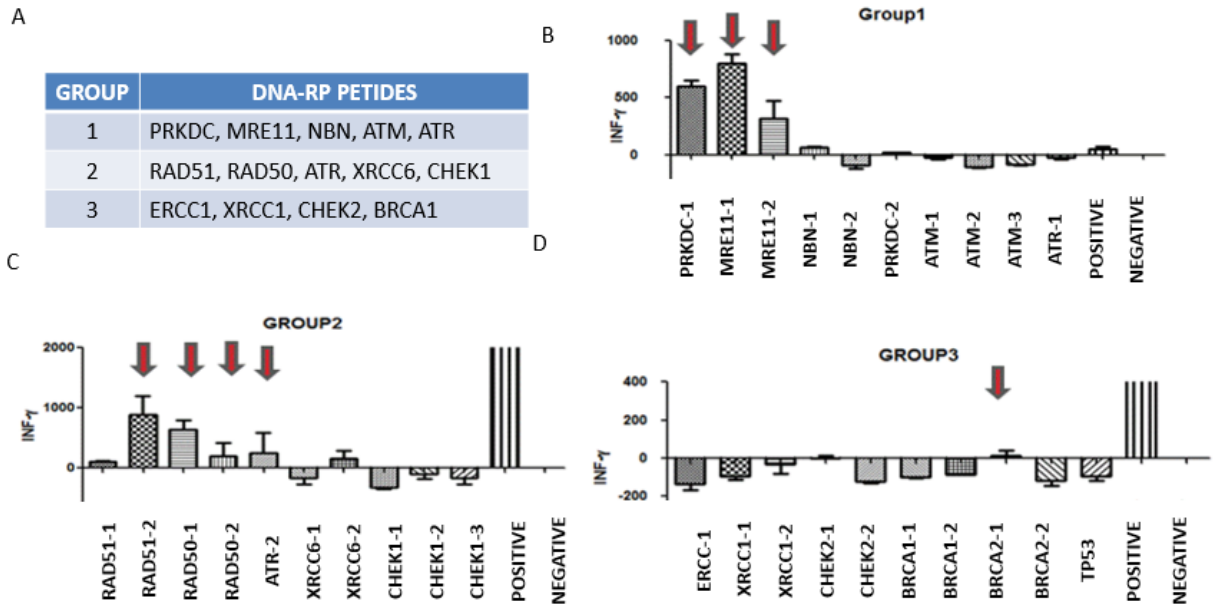


Figure 17. Analysis and Selection of Immunogenic DNA-RP-derived Peptide Epitopes for CD8+ T Cell Recognition.

A, Out of the 30 peptides synthesized (from **Table 4**), three groups of 10 peptides each were randomly generated. For each group of peptides, an equimolar peptide pool was generated, and each of the three mixtures of peptide loaded onto syngenic DC for use as a vaccine applied to naïve (non tumor-bearing) C57BL/6 mice. Overall, mice were vaccinated s.c. 3 times on a weekly basis with IL-12 gene-modified DC (DC.IL12, which promote $CD8^+$ T cell responses without the need for $CD4^+$ T helper epitopes pulsed with an equimolar pool of DNA-RP peptides (**Fig. 17A**). Control mice were vaccinated with DC.IL12 alone. One week after the final DC-based vaccine, the mice were euthanized and $CD8^+$ splenic T cells cultured with syngenic ($H\text{-}2^b$) antigen-presenting cell line EL4, or with EL4 cells that had been pre-loaded *in vitro* with individual DNA-RP-derived peptides (1 mM) after which, cell-free

supernatants were harvested and assessed for mIFN-γ concentration using a specific ELISA. Data are reported as mean ± SEM of triplicate determinations. (panel **B**-Group1, **C**-Group 2, **D**-Group 3) **.**

3.3.9.1 Mice vaccinated against DNA-RP developed CD8+ T cells reactive against specific DNA-RP peptides based on IFN-γ secretion as quantitated by ELISA

Amongst the peptides (**Table 4 in Methods**) analyzed in Group 1, $PRKDC_{1187-1194}$, $PRKDC_{620-628}$, $MRE11_{109-116}$, $MRE11_{208-216}$ proved to be most immunogenic, with $NBN_{273-281}$ also better recognized by T cells after specific vaccination. In group 2, the $RAD51_{58-66}$, $RAD51_{212-219}$, $RAD50_{1180-1187}$, $RAD50_{391-399}$, $ATR_{1390-1397}$, and $XRCC6_{483-491}$ were identified as immunogenic. In group 3, only the $BRCA2_{2848-2856}$ peptide displayed evidence of immunogenicity. Based on results obtained in 3 independently performed experiments, I selected the 9 most immunogenic peptides listed in **Table 5** (i.e., $RAD51_{58-66}$, $RAD51_{212-219}$ $RAD50_{1180-1187}$, $RAD50_{391-399}$, $ATR_{1390-1397}$, $PRKDC_{620-628}$, $MRE11_{109-116}$, $MRE11_{208-216}$ $NBN_{273-281}$) for use in subsequent studies involving combination chemoimmunotherapies. TMZ-inducible DNA-RP contains immunogenic peptides recognized by $CD8^+$ T cells in wild-type C57BL/6 mice.

Table 6. DNA-RP Peptides selected for Combination ACT.

DNA-RP	AA position	AA Sequence	Predicted H2-K^b or -D^b presentation
ATR	1390-1397	LSFAYGLL	K^b
"	200-208	MNVQNVEFI	D^b
PRKDC	1187-1194	LFYKFVPL	K^b
"	620-628	SALINLVEF	D^b
MRE11	208-216	NSWFNLFVI	D^b
"	109-116	VNYQDGNL	K^b
NBN	355-363	SAPVNMTTY	D^b
"	273-281	VGITNTQLI	K^b
RAD50	391-399	RQIKNFHEL	D^b
"	1180-1187	RNYNYRVV	K^b
RAD51	58-66	KELINIKGI	D^b
"	212-219	VESRYALL	K^b

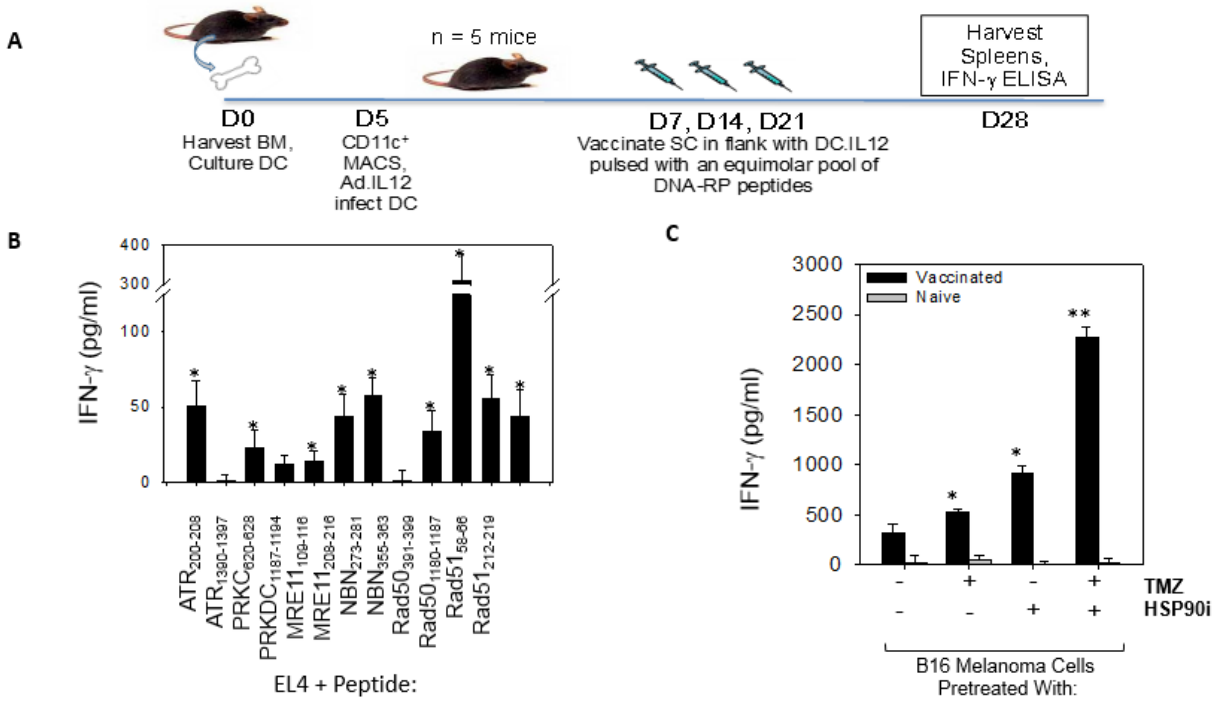


Figure 18. Vaccination against DNA-RP-derived Peptides Elicits Specific $CD8^+$ T Cells in Mice that Preferentially Recognize TMZ/HSP90i-conditioned B16 Melanoma Cells *In Vitro*.

To generate antigen-specific $CD8^+$ T cells reactive against DNA-RP, we first used a web-based algorithm to select for those peptides most likely to be coordinately proteasome-processed and MHC class I-presented in the $H\text{-}2^b$ C57BL/6 strain of mice (**Table 5**). Naïve mice were vaccinated s.c. a total of 3 times on a weekly regimen with syngenic DC.IL12 cells (i.e., DC engineered to produce mIL12p70 protein) that were loaded with an equimolar pool (1 μM for each peptide) of the chosen peptides (panel **A** schema, with additional detail in Materials and Methods). Splenic $CD8^+$ T cells isolated from vaccinated or control, naïve mice were then co-cultured at an effector-to-target cell ratio of 5:1 for 48h with EL4 ($H\text{-}2^b$, thymoma) cells pulsed (for 4h at 37°C) with individual DNA-RP peptides (1 μM, panel **B**), or against negative control EL4 cells, untreated B16 melanoma cells or B16 cells pre-treated with TMZ +/- HSP90i (panel **C**). Cell-free supernatants were then analyzed for mIFN-γ concentration using a cytokine-specific ELISA. Results are reflective of 3 independent experiments performed in each instance and are reported as mean ± SEM of triplicate determinations. *$p < 0.05$ vs. EL4 or B16 control cells.

3.3.9.2 Splenic T cells from mice vaccinated against pooled DNA-RP-derived peptides recognize B16 melanoma cells better after treatment with TMZ + HSP90i

As shown in **Fig. 18A**, mice vaccinated against DNA-RP developed $CD8^+$ T cells reactive against specific DNA-RP peptides based on IFN-γ secretion quantitated by ELISA. Amongst the peptides (**Table 6**) analyzed, the $RAD50_{1180\text{-}1187}$ peptide appeared most immunogenic in pooled vaccine formulations, with the peptides $ATR_{200\text{-}208}$, $PRKDC_{620\text{-}628}$, $MRE11_{109\text{-}116}$, $MRE11_{208\text{-}216}$, $NBN_{273\text{-}281}$, $RAD50_{391\text{-}399}$, $RAD51_{58\text{-}66}$ and $RAD51_{212\text{-}219}$ also recognized differentially by vaccinated vs. control $CD8^+$ T cells (**Fig. 18B**). In contrast, the peptides $ATR_{1390\text{-}1397}$, $PRKDC_{1187\text{-}1194}$ and $NBN_{355\text{-}363}$ failed to elicit statistically-significant, antigen-specific $CD8^+$ T cell responses in vaccinated mice, *$p < 0.05$.

Splenic T cells from mice vaccinated against pooled DNA-RP-derived peptides were also used as effector cells against target cells including the negative control EL4 thymoma, untreated B16 melanoma cells, and B16 melanoma cells pre-treated with TMZ (to upregulate DNA-RP)

+/- HSP90i (STA9090, to conditionally promote the loading of MHC class I complexes with DNA-RP-derived peptides generated via proteasome degradation). As shown in **Fig. 18C**, anti-DNA-RP-primed T cells, but not naïve T cells, were capable of preferentially recognizing B16 melanoma cells treated with TMZ + HSP90i.

3.4 SUMMARY

We found that melanoma cell lines are intrinsically refractory to the cytotoxic effects of TMZ, but that HSP90i could be used to sensitize these tumors to TMZ-induced apoptosis *in vitro*. When observing the cell cycle patterns of treated melanoma cells, sensitization might be the result of abrogating the transition from G1 to S phase and due to the reversal of G2 checkpoint loss caused by TMZ. Although TMZ was only a weak promoter of tumor cell apoptosis when used as a single-agent, this chemotherapy drug effectively stimulated melanoma cells to increase their expression of key DNA repair proteins involved in the G1 and G2/M cell cycle phases, including ATR, CHEK2, PRKDC, MRE11, NBN, RAD50 and RAD51. Since TMZ only promoted increased DNA-RP protein, but not transcript, expression, and we found that TMZ treatment led to augmented transcription of HSP90, we hypothesize that TMZ-induced HSP90 may be stabilizing DNA-RP client proteins. This would lead to the prediction that the HSP90i destabilize tumor cell expression of these DNA-RP, making them more susceptible to the cytotoxic action of TMZ (as we observed in combined experiments). We also determined that mice vaccinated against DNA-RP developed $CD8^+$ T cells reactive against RAD50, RAD51, PRKDC, MRE11, NBN and ATR. These peptides enabled us to assess combination

chemoimmunotherapy (TMZ +/- HSP90i +/- DNA-RP targeted immunotherapy) for melanoma-bearing mice.

Proposed schema for improved recognition of TMZ/HSP90i-conditioned melanoma cells by therapeutic anti-DNA-RP CD8$^+$ T cells. Melanoma cells, even if briefly treated with TMZ, upregulate expression of DNA-RP that represent clients of the HSP90 chaperone complex. Subsequent administration of HSP90i (such as STA9090/Ganetespib) results in the proteasome-dependent degradation of DNA-RP, providing a conditional source of peptides for presentation in MHC class I complexes on the melanoma cell surface. Once expressed on the tumor cell surface, these complexes are capable of being recognized by DNA-RP-reactive CD8$^+$ T cells that may be induced *in vivo* by active, specific vaccination and applied in ACT-based treatment approaches.

4.0 HSP90 INHIBITION ENHANCES THE ANTI-TUMOR EFFICACY OF COMBINATION CHEMOIMMUNOTHERAPY TARGETING DNA REPAIR PROTEINS

4.1 ABSTRACT

Although melanoma is generally considered to be resistant to genotoxic chemotherapeutic agents, we report that treatment with Temozolomide (TMZ) induces murine and human melanoma cell lines to upregulate expression of DNA repair proteins (DNA-RP) that represent HSP90 clients *in vitro* and *in vivo*. TMZ-induced DNA-RP can then serve as protein substrates for proteasome-dependent generation of MHC class I-presented peptide epitopes upon subsequent administration of the HSP90 inhibitor STA9090 (Ganetespib). Notably, pre-conditioning of progressively growing B16 melanomas in C57BL/6 mice with systemic TMZ + STA9090 treatment sensitizes melanoma cells to the anti-tumor action of adoptively-transferred DNA-RP-specific $CD8^+$ T cells. The efficacy of combination chemoimmunotherapy integrating TMZ, STA9090 and antigen-specific adoptive T cell transfer was associated with superior levels of Type-1 $CD8^+$ TIL in treated tumors and more expansive areas of tissue apoptosis within the tumor microenvironment. These data support the possible translation of such combination treatment strategies into the clinic for the treatment of patients with melanoma.

4.2 INTRODUCTION

Melanoma is the 5th most common form of cancer in the United States and is one of three cancer types that continue to increase in incidence (Siegel et al., 2013). Metastatic melanoma, the most dangerous form of skin cancer, responds poorly to conventional chemotherapy, with a median overall survival of less than one year (Guida et al., 2012). Although the results of several recent clinical trials support the potent anti-melanoma activity of immune checkpoint inhibitors (i.e., anti-CTLA4, anti-PD1 or anti-PD-L1 antibodies) as standard of care, few patients receive durable treatment benefit (Phan et al., 2003; Brahmer et al., 2010; Cohen & Sznol, 2015). As a consequence, there remains a clear need for the continued development of novel, effective second-line therapy options for such patients.

Chemotherapeutic agents, such as TMZ, cause “incorrectable” DNA lesions in treated tumor cells, most commonly DNA double-strand breaks (Fojo, 2001; Luqmani, 2005; Khan et al., 2011). Moderately-impacted tumor cells may amplify gene products associated with DNA repair in order to maintain cellular viability, leading to a state of acquired chemotherapy resistance (Kauffmann et al., 2008). In the melanoma setting, DNA-RP (such as ATM, CHEK2, ATR, CHEK1, BRCA1, BRCA2, MRN complex proteins (MRE11, RAD50, NBN), XRCC6, XRCC5, RAD51, PRKDC) are transcriptionally overexpressed in a disease stage-associated manner in association with chemotherapy-resistance and poor overall survival (Schmidt-Ullrich et al., 2003; Dote et al., 2006; Jewell et al., 2010; Taipale et al., 2010; Ha et al., 2011; Stecklein et al., 2012; Guida et al., 2012; Ko et al., 2012; Che et al., 2013; Acquaviva et al., 2014; Pennisi et al., 2015).

Notably, tumor cell overexpression of DNA-RP is also sustained post-translationally via the stabilizing action of chaperone molecules, such as heat shock protein-90 (Dote et al., 2006;

Taipale et al., 2010; Ha et al., 2011; Ko et al., 2012; Stecklein et al., 2012; Che et al., 2013; Acquaviva et al., 2014;), a highly-abundant molecule in the cancer proteome (Whitesell & Lindquist, 2005). HSP90 forms the core of a super-chaperone machine consisting of HSP70, HSP40, HIP and HOP, which extends the molecular lifespan of a growing list of client proteins, including signaling protein kinases, transcription factors, DNA-RP and other cytosolic or nuclear proteins in their functionally mature and active conformations (Taipale et al., 2010). While most targeted therapy agents antagonize 1-2 individual proteins or signaling pathways, which might lead to the selection of compensatory pro-oncogenic pathways by adapting tumor cells, HSP90i would be expected to coordinately disrupt multiple DNA-RP clients (and a range of pro-tumor signaling pathways), supporting the survival/progression of heterogeneous populations of cancer cells (Koga et al., 2009). Since HSP90i are known to promote the conditional degradation of client proteins via the proteasome pathway (Whitesell & Lindquist, 2005; Taipale et al., 2010; Acquaviva et al., 2014), a major conduit for immunogenic peptides presented by MHC class I molecules to $CD8^+$ T effector cells (Goldberg et al., 2002), we reasoned that melanoma cells conditioned by genotoxic chemotherapy agents and then treated with HSP90i might represent preferred targets for DNA-RP-specific $CD8^+$ T cells *in vivo*.

Data developed in both murine and human melanoma models suggest that tumor expression of DNA-RP is upregulated in the presence of TMZ, and that tumor overexpressed DNA-RP can then be induced to undergo proteasome-dependent degradation after treatment with the HSP90i STA9090, in support of the superior anti-tumor efficacy of adoptively-transferred $CD8^+$ T cells specific for DNA-RP that constitute HSP90 client proteins. The anti-tumor benefit of the combination immunotherapy was associated with enhanced Type-1 T cell infiltration into tumor lesions, with higher levels of tumor cell apoptosis *in vivo*, and with the upregulated

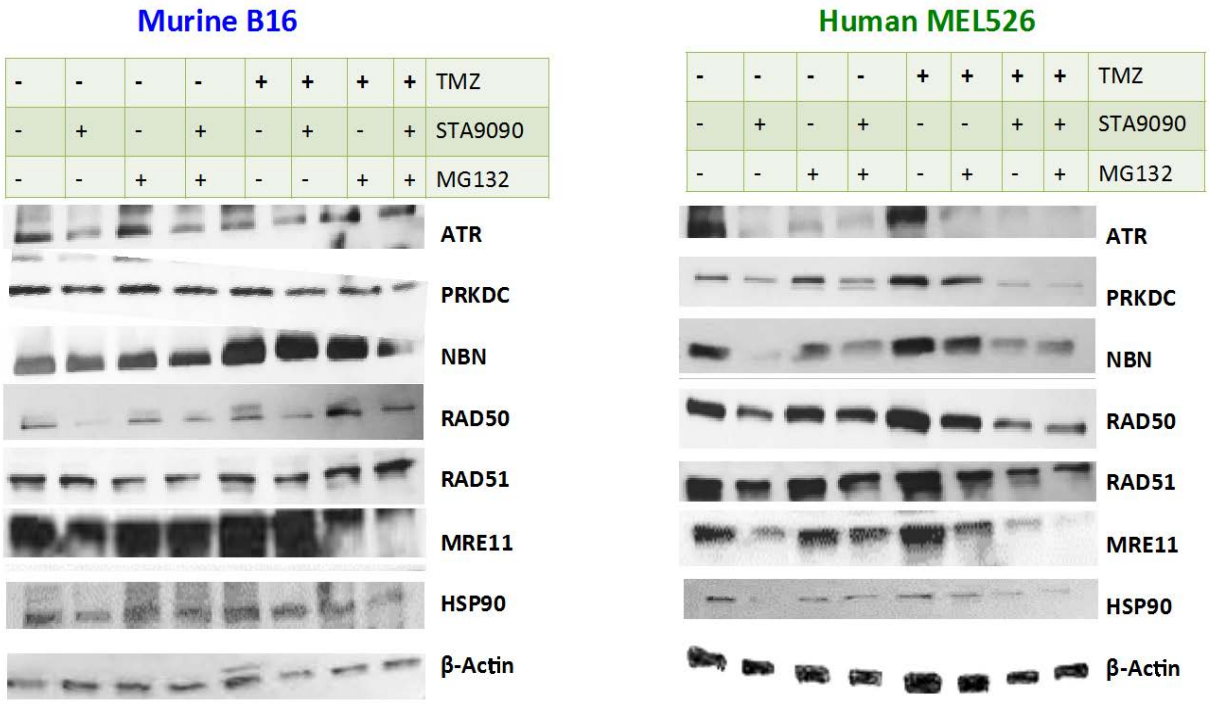
expression of inflammation-responsive PD-L1 by tumor/stromal cells in therapeutic tumor microenvironment. Overall, these studies serve as a foundation for the design and implementation of novel chemo-immunotherapeutic strategies for translation into the clinic for the treatment of patients with advanced-stage melanoma or alternate cancer types that exhibit intrinsic or developed resistance to genotoxic chemotherapies.

4.3 RESULTS

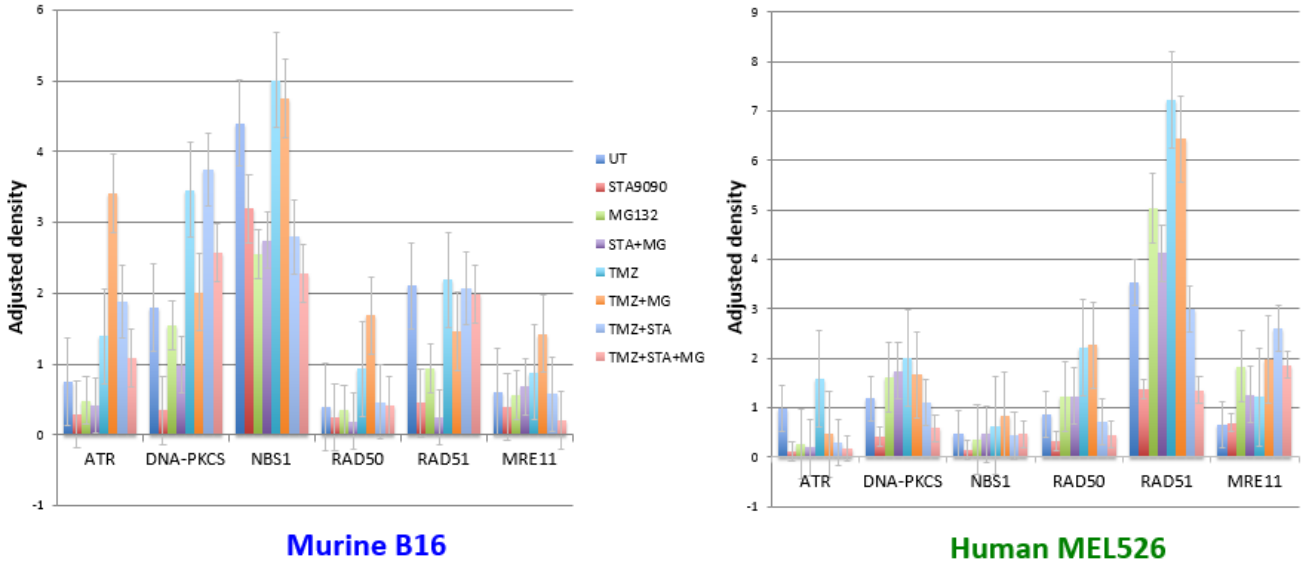
4.3.1 DNA-RP overexpression in TMZ-conditioned melanomas is stabilized by HSP90 and subject to proteasome-dependent degradation in the presence of HSP90i

Since members of the DNA-RP family of proteins have been previously suggested to represent HSP90 clients, we next analyzed whether expression of DNA-RP by wild-type and TMZ-conditioned B16 (murine) and Mel526 (human) cells was sensitive to treatment with the HSP90i STA9090 *in vitro*. As shown in **Fig. 19**, we found that both intrinsic and TMZ-induced DNA-RP expression was strongly downregulated after co-culture with STA9090, with these effects partially antagonized by the inclusion of the proteasome inhibitor MG132. These data support the conclusion that at least a fraction of melanoma DNA-RP undergo proteasome-dependent degradation when treated with HSP90i, theoretically providing a therapy-associated source of MHC class I presented peptides for enhanced $CD8^+$ T cell recognition.

A


Murine B16
Human MEL526
TMZ
STA9090
MG132
ATR
PRKDC
NBN
RAD50
RAD51
MRE11
HSP90
β-Actin

B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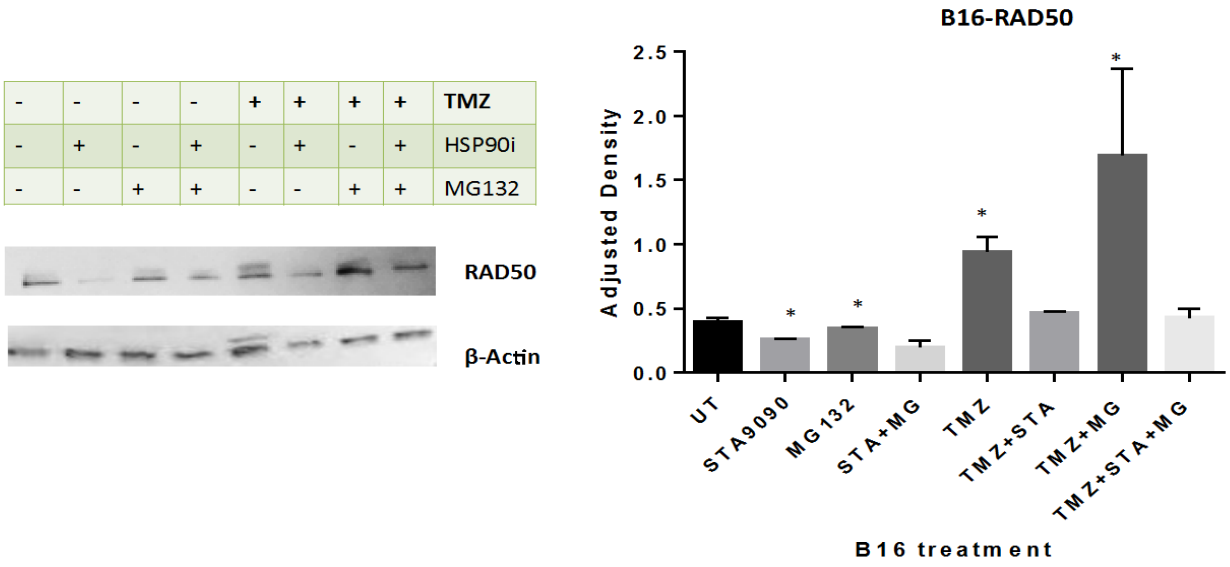
MRE11
Murine B16
Human MEL526

C



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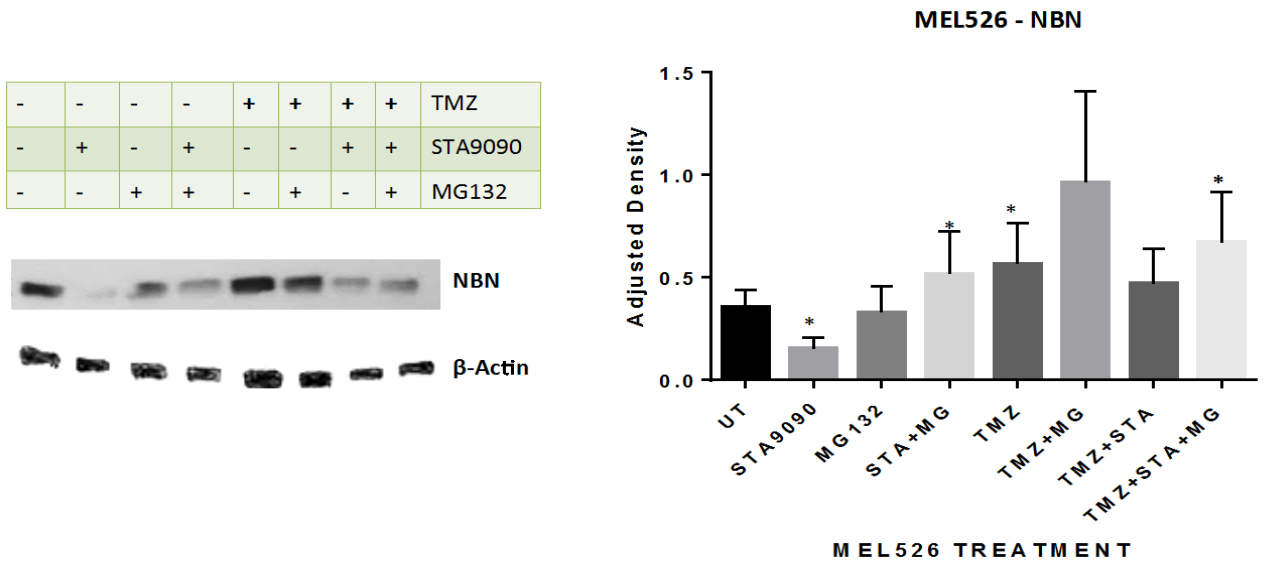
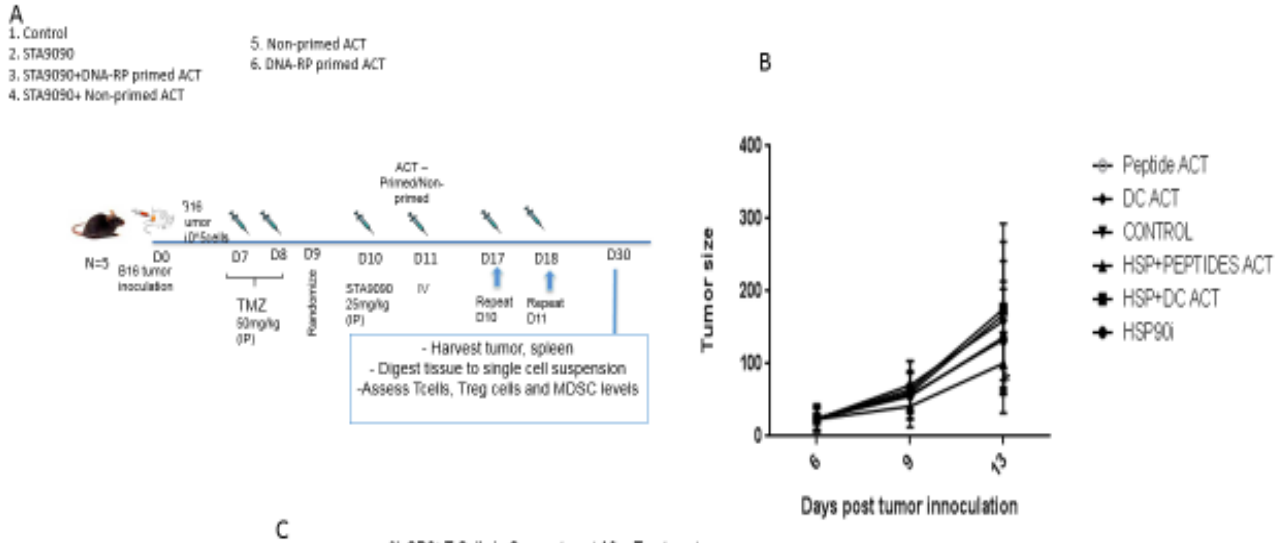


Figure 19. TMZ-upregulated DNA-RP in Murine and Human Melanoma Cell Lines are Degraded in the Presence of HSP90i STA9090 in a Proteasome-dependent Manner.

Murine B16 and human Mel526 melanoma cells were pre-treated with TMZ (50 μM) for 3h at 37ºC, before subsequent culture in the absence or presence of HSP90i (50 μM) and/or MG132 (10 μM) at 37ºC for an additional 12h. Cells were then solubilized and DNA-RP proteins analyzed by Western Blotting panel **A**. Specific DNA-RP

protein bands in blots were quantitated (panel **B**) by densitometry scanning and normalized to control β–actin as described in the Materials and Methods and is reported as mean ± SEM**.** Examples of treatment changes in DNA-RP expression are provided for RAD50 in the B16 (panel **C**) and NBN in Mel526 (panel **D**) melanoma cell lines. Results are reflective of three independent experiments. *p < 0.05.



C

% CD8⁺ T Cells in Compartment After Treatment

	UT	Control VAC	HSP90i	DNA-RP VAC	Control VAC + HSP90i	DNA-RP VAC + HSP90i
Spleen	50.4	69	47.8	72.9	43.2	61.9
Tumor	0.2	0.2	0.1	0.6	0.6	3.4

Figure 20. Combination TMZ/HSP90i + DNA-RP Primed ACT Provides Superior Anti-Tumor Efficacy vs. either Chemo- or Immune-Monotherapy.

As depicted in the panel **A** schema, C57BL/6 mice were injected s.c. in their right flanks with 10^5 B16 melanoma cells and tumor allowed to establish for 6 days. All animals were conditioned with TMZ (50 mg/kg) for 2 days, before then randomizing them into 5 mice/cohort (i.e., each group with a comparable mean tumor size of approximately 40-50 mm^2) on day 8. Tumor-bearing mice were then treated with HSP90i (STA9090; 25 mg/kg, i.p., on days 8 and 15) or vehicle control (DMSO) +/- ACT (10^6 splenic $CD8^+$ T cells non-primed or primed with DNA-RP peptides injected i.v. into the tail vein on days 9 and 16). Tumor growth was then monitored every 3-4 days through day 30 (euthanasia) and is reported as mean ± SD tumor size in mm^2 (**B**); *p < 0.1 vs. all other cohorts

(ANOVA and area under the curve). Animals bearing day 13 tumors of median size were subsequently harvested and analyzed by flow cytometry (panel **C**, for $CD8^+$ TIL) as outlined in the Materials and Methods.

4.3.2 Combined TMZ/HSP90i + anti-DNA-RP vaccination provides superior anti-melanoma efficacy vs. single modality treatment *in vivo*

Based on the operational paradigm that TMZ + HSP90i treatment results in upregulation of DNA-RP followed by enhanced processing and MHC class I presentation of DNA-RP-derived peptides, we next evaluated whether this conditional regimen would facilitate the anti-tumor action of ACT using DNA-RP-specific $CD8^+$ T cells induced by syngeneic DC pulsed with immunogenic DNA-RP-derived peptides that had previously been identified in **Fig. 18**. Tumor-bearing mice were treated as shown in **Fig. 20A**, and then monitored for tumor growth over time, with animals harboring median-sized tumors euthanized on day 30 for assessment of immune cell infiltration. We observed that the group treated with TMZ/HSP90i + ACT had the slowest growing tumors (**Fig. 20B**) and the highest degree of $CD8^+$ T cell infiltration into tumors (**Fig. 20C**) when compared to all other cohorts of treated animals.

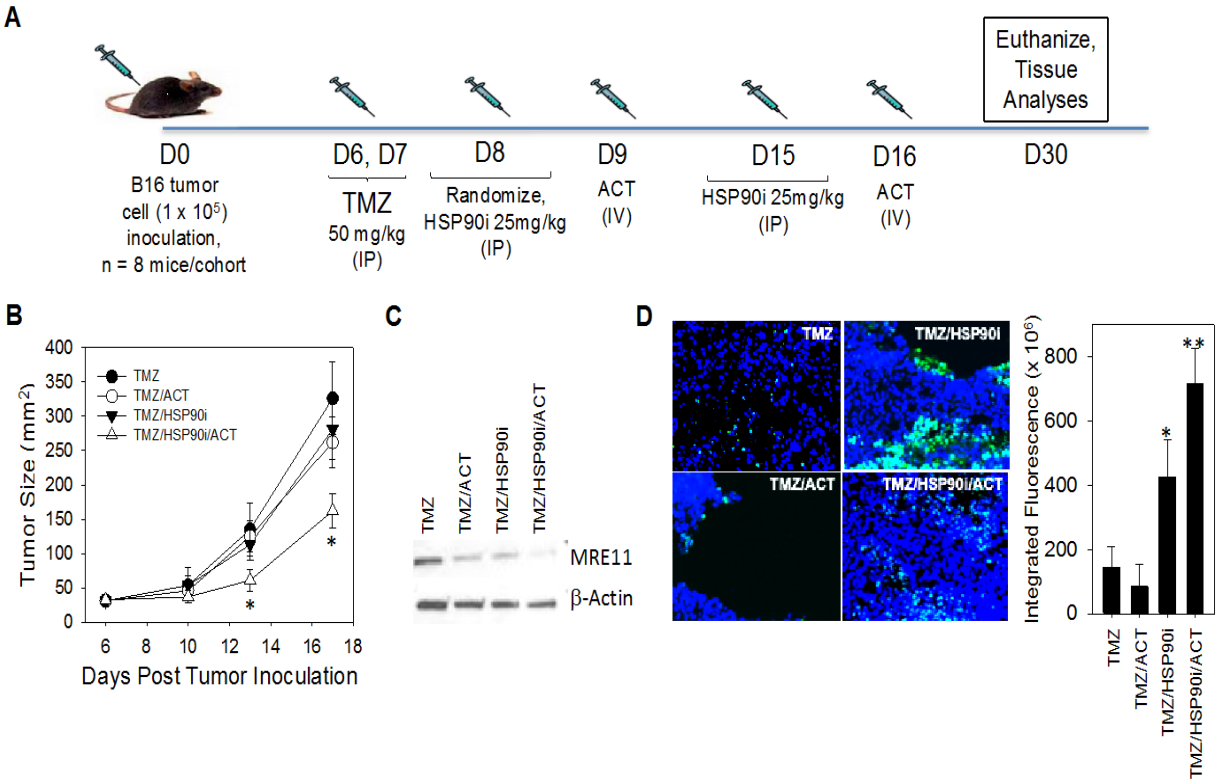


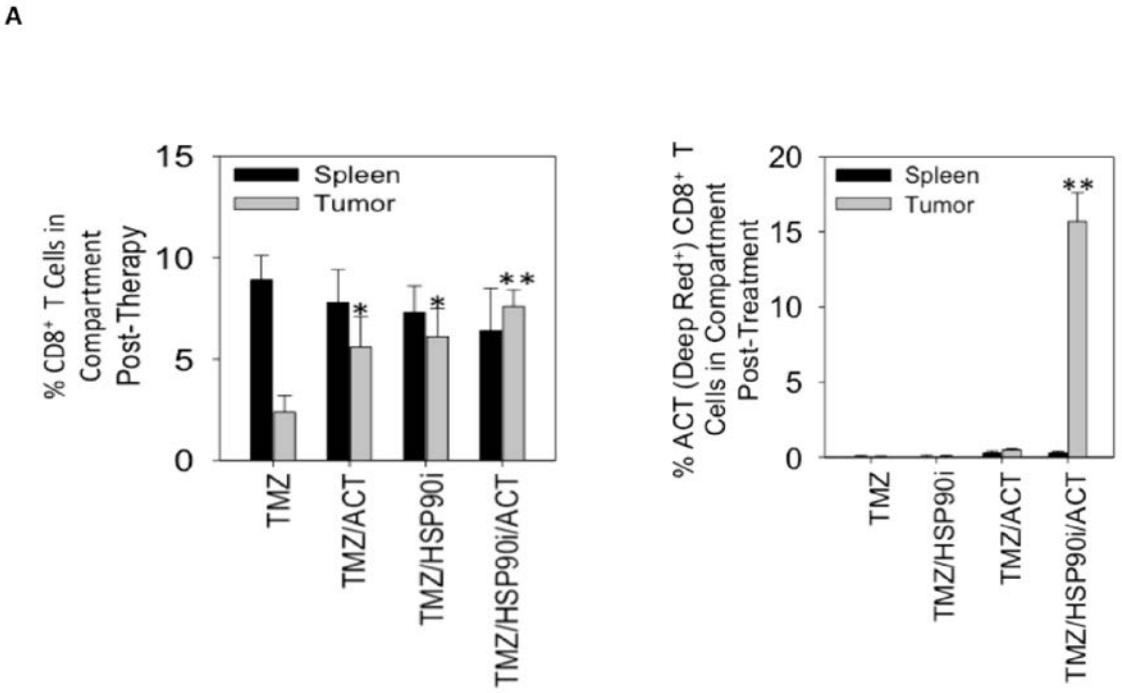
Figure 21. Combined TMZ/HSP90i + ACT Therapy of Established B16 Melanomas is Superior to Chemo- or ACT-Monotherapy in Reducing Tumor Growth *In Vivo*.

As depicted in the panel **A** schema, C57BL/6 mice were injected s.c. in their right flanks with 10^5 B16 melanoma cells and tumor allowed to establish for 6 days. All animals were conditioned with TMZ (50 mg/kg) for 2 days, before then randomizing them into 8 mice/cohort (i.e., each group with a comparable mean tumor size of approximately 40-50 mm^2) on day 8. Tumor-bearing mice were then treated with HSP90i (STA9090; 25 mg/kg, i.p., on days 8 and 15) or vehicle control (DMSO) +/- ACT (10^6 splenic $CD8^+$ T cells harvested from DNA-RP peptide vaccinated mice as in Fig. 18A injected into the tail vein on days 9 and 16). Tumor growth was then monitored every 3-4 days through day 30 (euthanasia) and is reported as mean $\pm$ SD tumor size in mm^2 (**B**); *$p < 0.05$ vs. all other cohorts (ANOVA and area under the curve). Animals bearing day 30 tumors of median size were subsequently harvested and analyzed for expression of DNA-RP MRE11 by Western Blotting (**C**) and for cellular apoptosis by TUNEL staining as imaged and quantitated and is reported as mean $\pm$ SEM, using fluorescence microscopy and Metamorph software, per the Materials and Methods (**D**). *$p < 0.05$ vs. TMZ alone; **$p < 0.05$ vs. all other cohorts (ANOVA). All results are reflective of three independent experiments performed in each instance.

4.3.3 Combination immunotherapy using TMZ/HSP90i + ACT using $CD8^+$ T cells reactive against DNA-RP provides superior anti-tumor efficacy versus component monotherapies

Given the ability of TMZ to enhance melanoma expression of DNA-RP and for the HSP90i STA9090 to promote the subsequent proteasome degradation of these client proteins, we hypothesized that such pre-conditioning would sensitize progressively growing B16 melanomas to the anti-tumor action of adoptively-transferred $CD8^+$ T cells reactive against DNA-RP-derived peptide epitopes. For these experiments, B16 melanomas were established s.c. in C57BL/6 mice for 6 days, at which time they received i.p. TMZ conditioning for 2 consecutive days, followed by weekly i.p. administration of STA9090 (25 mg/kg; or the DMSO vehicle control) beginning on days 8 and 15 post-tumor inoculation (**Fig. 21A**). One day following treatment with the HSP90i or DMSO (i.e., days 9 and 16 post-tumor inoculation), the mice received i.v. injection of 1 x 10^6 fluorescently (Deep red dye)-labeled anti-DNA-RP $CD8^+$ T cells (isolated from the spleens of naïve syngenic mice vaccinated against pooled DNA-RP peptides as in **Fig. 21**) in their tail veins. As shown in **Fig. 21B**, while treatment of TMZ-conditioned B16-bearing mice with either STA9090 alone or anti-DNA-RP $CD8^+$ T cells alone failed to impact tumor growth, the combined therapy (STA9090 + adoptive cell therapy; ACT) resulted in significantly reduced tumor progression. As expected, therapeutic intervention with TMZ/STA9090 (+/- ACT) led to a reduction in day 17 tumor expression of DNA-RP (such as MRE11) *in vivo* (**Fig. 21C**). Interestingly, the combined TMZ + ACT treatment protocol also resulted in less MRE11 expression in tumors (when compared to the TMZ only treatment cohort of mice (**Fig. 21C**), possibly due to immune selection by vaccine-induced anti-MRE11 $CD8^+$ T cells used for ACT.

We also observed the greatest degree of cellular apoptosis within tumors of mice treated with TMZ/HSP90i + ACT (**Fig. 21D**; $p < 0.05$ vs. all other cohorts, ANOVA).



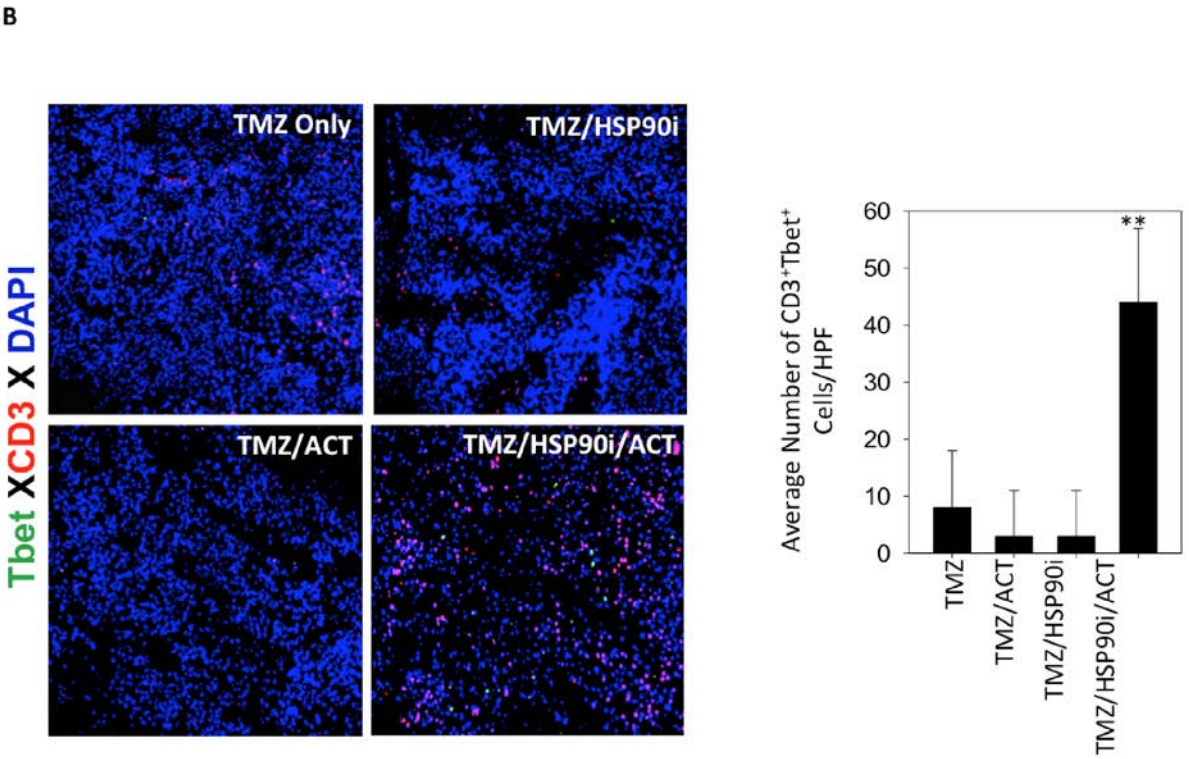


Figure 22. Combined TMZ/HSP90i + ACT Therapy of Established B16 Melanomas Promotes Superior Tumor Infiltration by Type-1 CD8$^+$ TIL.

Day 30 harvested tumors were analyzed by flow cytometry (panel **A**, for CD8$^+$ TIL and Deep red dye$^+$ CD8$^+$ TIL) or fluorescence microscopy (**B**, for Tbet$^+$CD3$^+$ TIL) as outlined in Materials and Methods. Results are reflective of 3 independent experiments performed and all error bars reflects mean $\pm$ SEM. **p < 0.05 vs. all other cohorts (ANOVA).

4.3.4 Combined TMZ/HSP90i + specific ACT therapy of established B16 melanomas promotes superior tumor infiltration by Type-1 CD8$^+$ TIL

Additional analyses of day 17 melanoma tissues using flow cytometry and immunofluorescence microscopy revealed that combined TMZ/STA9090 + anti-DNA-RP T cell-based ACT was associated with the superior infiltration of tumors by Deep red dye$^+$ ACT T cells (**Fig. 22A**) and Tbet$^+$CD8$^+$ T cells (**Fig. 22B**). In groups treated with STA9090, higher levels of CD8$^+$ T cells

were observed in tumors vs. spleens, suggesting that HSP90i facilitates tumor infiltration by T cells, with similar results observed in experiments using Deep red dye^{+} ACT T cells. The combination of HSP90i and ACT led to a >30-fold increase in labeled TIL vs. treatement with ACT alone, supporting a role for STA9090 in improved tumor infiltration by CD8^{+} T cells.

4.4 DISCUSSION

The major novel findings in Chapter 4 are that DNA-RP expressed by melanoma cells can be upregulated by genotoxic agents, such as TMZ, and can then be conditionally-induced to undergo proteasome-dependent degradation by HSP90i, leading to improved recognition of melanoma cells by therapeutic anti-DNA-RP specific CD8^{+} T cells *in vitro* and *in vivo*. As a consequence, despite the known inherent resistance of human melanomas to TMZ (Alvino et al., 2006; Mhaidat et al., 2007; Rietschel et al., 2008) which we also observed *in vivo* in B16 models, in Chapter 3), this agent can be used to "accumulate" higher levels of DNA-RP to serve as a supply of immunogenic peptides in combination therapies integrating HSP90i such as STA9090. This effect was not restricted to TMZ, as we also observed that the genotoxic anthracycline Doxorubicin (Supino et al., 1988; Ramachandran et al., 1993), but not the BRAFi Dabrafenib, was also able to promote elevated expression of DNA-RP in treated melanoma cells that were consequently susceptible to HSP90i-induced degradation.

The finding that we could readily induce autoimmune CD8^{+} T cell responses against non-mutated "self" peptides derived from DNA-RP using DC-based vaccines is consistent with previous reports supporting the ability of melanoma lineage antigen (MART1, PMEL, tyrosinase and others)-based vaccines to activate a low-to-moderate avidity T cell repertoire in mice and

humans, despite the host's apparent operational "self-tolerance" to such antigens (Schreurs et al., 2000; Slingluff et al., 2006; Rao et al., 2012). Although we only analyzed the capacity of combination TMZ/HSP90i-based therapy to improve the anti-tumor efficacy of adoptively-transferred anti-DNA-RP $CD8^+$ T cells in our melanoma treatment modeling, it would be anticipated that this drug combination would also improve the therapeutic benefits associated with active specific vaccination against DNA-RP (using antigen-loaded Type-1-polarized DC or alternate strategies). Such analyses will be pursued in future studies extended to include alternate genotoxic agents (including DOX known to promote tumor "immunogenic cell death" (Obeid et al., 2007)) and additional inhibitors of HSP90 (including alternate in-clinic HSP90i that affect ATP-binding (Butler et al., 2015), as well as, HDACi known to inhibit HSP90 function via altered acetylation of regulatory lysine residues (Bali et al., 2005; Scroggins et al., 2007; Kekatpure et al., 2009). Although no evidence for pathologic autoimmunity was observed in our ACT modeling, it will be important to monitor mice vaccinated against DNA-RP in future experiments for deleterious autoimmune sequelae, particularly in combination approaches designed to promote strong inflammatory responses (Gilboa, 2001; Phan et al., 2003; Kong et al., 2009; Brahmer et al., 2012; Weber et al., 2015).

Our findings also support the ability of combined TMZ/HSP90i + ACT-based therapy to promote enhanced infiltration of melanomas by Type-1 ($Tbet^+$) $CD8^+$ T cells (including the ACT population of deep red dye-labeled $CD8^+$ T cells) in association with improved treatment outcome, and are consistent with several recent reports supporting higher numbers of $CD8^+$ TIL as a predictive biomarker for patient benefit to immunotherapy (Rusakiewicz et al., 2013; Berghoff et al., 2015; Noble et al., 2016; Tokito et al., 2016). However, $CD8^+$ T effector TIL produce IFN-γ, which can promote upregulated expression of immune checkpoint molecules

such as PD-L1 (Mandai et al., 2016; Tokito et al., 2016) in the TME. PD-L1 agonism of PD-1 on TIL can reduce the viability and anti-tumor functionality of these protective T cell populations *in vivo* (Brahmer et al., 2012). Hence, future studies should be designed to determine whether the anti-PD-L1 and/or anti-PD-1 immune checkpoint inhibitors can further improve the anti-tumor efficacy of our current combination chemoimmunotherapy approach.

The ability of HSP90i to enhance recognition of melanoma target cells by $CD8^+$ T cells might yet be suboptimal, since cellular expression of the HSP70 chaperone molecule is known to be increased in response to HSP90i (Whitesell et al., 2003), and HSP70 can substitute for HSP90 in stabilizing client proteins (Cavanaugh et al., 2015). Such complementation in the molecular salvage pathway might limit the pool of DNA-RP accessible for proteasome processing, and hence, the amount of DNA-RP-derived peptides capable of being presented to anti-DNA-RP $CD8^+$ T cells. As a consequence, it might be anticipated that transient coordinate application of HSP70i + HSP90i might result in more robust anti-tumor activity mediated by DNA-RP-specific T effector cells, as well as, to a greater degree of tumor cell apoptosis based on reduced levels of functional DNA-RP in treated tumor cells. Despite concerns for systemic toxicities that might arise in such combination regimens, these treatment protocols will be carefully evaluated in future studies.

In conclusion, we believe that these translational results support the design of new chemoimmunotherapeutic options for patients with melanoma or alternate advanced-stage forms of solid cancer that exhibit chemotherapy-resistance and/or that fail current first-line therapies (including immune checkpoint blockade).

5.0 DISCUSSION AND SUMMARY

Cancer remains a major public health threat worldwide. Estimated new cancer cases diagnosed in the US in 2016 alone will be 1,685,210. Overall cancer-related deaths in the US in 2016 are expected to be 595,690, making cancer the second leading cause of death in this country, exceeded only by heart disease (Cancer Facts and Figures, 2016). Although there was ~20% improvement in 5-year relative survival rate for all cancers diagnosed from 2005-2011 (69%) in comparison to 1975-1977 (4%) due to early diagnosis of certain cancers and improvements in targeted therapeutics, there remains a critical need to develop more effective treatment options for patients afflicted with cancer. In this regard, even though the combination chemoimmunotherapy that we developed was applied to melanoma models, it is clearly applicable to virtually any form of cancer based on the essential nature of DNA-RP (and HSP90) to general tumor cell survival/progression. Having said this, melanoma is a common disease that accounts for 75% of skin cancer-related deaths in the US each year. The incidence of cutaneous melanoma has been steadily increasing over the past three decades, primarily amongst adults over 50 years of age (Cancer Facts and Figures, 2016; Siegel et al., 2016). Like other forms of cancer, melanoma has evolved numerous mechanisms that confer resistance to conventional cancer therapeutic, making melanoma a perfect model in which to test the hypotheses of this study.

Despite recent advancements in cancer immunotherapy and targeted therapies, many patients worldwide still receive chemotherapeutic agents as first-line treatment. Chemotherapy has been used extensively in the setting of cancer treatment since the 1940s. Seventy years later, however, such therapies have not demonstrated profound survival benefits when applied as single agents, due to the rapid development of treatment-refractory disease. Hence, the vast majority of drug-resistant patients require effective second-line therapies that are capable of conferring improved clinical benefits (Fojo, 2001; Chabner & Roberts, 2005). A patient's tumor mass represents a highly heterogeneous population of malignant cells, with only a subset of these cells typically sensitive to the action of a given monotherapy. Treatment-resistant tumor clones readily persist and expand, making any initial therapeutic benefits exceedingly transient in nature, with progressive tumors likely to exhibit even more aggressive characteristics (i.e., invasion, metastasis, immune suppressive capacity (Persidis, 1999; Hanahan & Weinberg, 2011; Rebucci & Michiels, 2013).

Tumors adopt several mechanisms to resist the cytotoxic action of chemotherapeutic agents. These include the accumulation of activating mutations of oncogenes or inactivating mutations of tumor suppressor genes, increased drug export pathways to lower intracellular drug concentrations, limiting or circumventing programmed cell death pathways to evade apoptosis, and by taking advantage of the DNA damage response (Rebucci & Michiels, 2013). Amongst many potential mechanisms underlying cancer resistance to genotoxic chemotherapy agents, overexpression of DNA repair proteins has been identified as a dominant player. All conventional chemotherapeutic drugs induce tumor cell death by causing accumulated DNA damage that eventually leads to apoptosis. Such therapy-induced genetic alterations can be counteracted by the action of "normal" DNA repair pathways that are accentuated in cancer cells

based on the intrinsic overexpression of DNA-RP (Yoshimoto et al., 2012). A study assessing the genetic profile of primary melanomas progressing to metastatic disease over a 4-year period documented a striking correlation with upregulated expression of DNA-RP in cancer cells. Furthermore, such overexpression of DNA-RP has been correlated to the resistance of metastatic melanoma to both chemo- and radiotherapy (Kauffmann et al., 2008). Such clinical associations have led to the targeting of DNA repair pathways as a therapeutic strategy in the cancer setting (Fojo, 2001).

Many small molecule inhibitors of DNA repair pathways have been developed as cancer therapeutics. PARP-inhibitors have been evaluated in phase I/II clinical trials either as a monotherapy or in combination with TMZ or other genotoxic drugs (Helleday et al., 2008). Though PARP is involved in base excision repair, it is not solely involved in DNA repair. There are other clinical trials involving small molecule inhibitors targeting proteins that are solely involved in the process of DNA repair. Beyond PARP1 as a therapeutic target in the base excision repair pathway, inhibitors targeting APE1, FEN1 are currently under investigation in translational/clinical models. Mismatch repair (MMR) pathway inhibitors targeting MLH1 and MSH2, as well as, Nucleotide Excision repair inhibitors targeting multiple proteins within the pathway (such as XPA, RPA) are currently under investigation as well (Kelley et al., 2014). For more lethal DSB lesions, alkylating agents such as TMZ (which modifies nitrogen atoms in the DNA ring causing a mismatch repair that is not repaired by MMR) is being applied to induce tumor cell death by provoking DSB. Indeed, the status of DSB repair activity appears to determine the comparative anti-tumor efficacy of genotoxic chemotherapy drugs (Yoshimoto et al., 2012). Small molecule inhibitors are being developed to target DSB repair pathway proteins, including RAD51and RPA in HR, in addition to PRKDC, PNKP, and DNA-Ligase 4 in NHEJ

(Tan & Lynch, 2013; Kelley et al., 2014). Even though translational research and clinical trials have validated the therapeutic effectiveness of specific DNA-RP inhibitors, the utility of these agents becomes more challenging as the disease stage progresses and the TME becomes increasingly hypoxic, making cancer cells more prone to DNA mutations linked to chemotherapy-resistance (Helleday et al., 2008; Kinsella, 2009). This suggests that the action of genotoxic chemotherapies will be optimized under conditions that coordinately counteract multiple tumor DNA repair pathways and replicative stressors in the TME (i.e., hypoxia).

This is where HSP90 inhibitors come into play. HSP90 along with other molecular co-chaperones have hundreds of cellular client proteins that play roles in many of the "hallmarks of cancer" first set forth by Hanahan and Weinstein (Hanahan & Weinstein, 2000) including self-sufficiency in growth signals, insensitivity to growth-inhibitory mechanism, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2011; Miyata et al., 2013). Antagonizing HSP90 function in tumor cells is an "economical" therapeutic option, as this chaperone molecule is preferentially overexpressed in cancer cells where it coordinately stabilizes a number of DNA-RP. This provides confidence for the safety profile of HSP90i in cancer patients and for the ability of these agents to interrupt the evolution of treatment-refractory disease amongst heterogeneous tumor cell populations. Notably, HSP90 expression levels in tumor cells have been reported to be prognostic of decreased survival in breast cancer patients (Pick et al., 2007). Another study showed an induction of HSP90 expression in malignant melanoma and melanoma metastasis (Becker et al., 2004). Furthermore, the authors reported that the activity of HSP90 in the TME is increased via post-translational modification, with tumor associated antigens

exhibiting higher ATPase activity (Kamal et al., 2003; Becker et al., 2004; Barrott & Haystead, 2013).

HSP90 inhibitors continue to be investigated in the clinical setting, with 2nd-generation small molecule HSP90i, such as STA9090 and NVP-AUY922 demonstrating superior anti-tumor effectiveness when compared to 1st-generation geldanamycin (GA)-based drugs. In contrast to GA, 2nd-generation agents are not confounded by cardiovascular and hepatic toxicity issues. Furthermore, these newer agents (such as STA9090) have over 100-fold greater affinity for tumor- vs. normal cell-associated HSP90 complexes, providing drug-selectivity for diseased tissue (Kamal et al., 2003; Wang et al., 2010). Interestingly, STA9090 appears to exhibit greater anti-tumor activity when compared with GA as a single modality therapy, and a randomized phase II study of STA9090 administered in combination with Docetaxel (an anti-mitotic chemotherapy drug) as second-line therapy for advanced non-small-cell lung cancer patients showed favorable progression-free survival and overall survival in the adenocarcinoma population and significant benefit in a subgroup of patients with advanced-stage disease (Ramalingam et al., 2015). Such findings clearly support the utility of the HSP90i, STA9090 to combat chemotherapy-refractory disease.

ACT has shown remarkable clinical success for cancer therapy in the setting of hematopoietic malignancies, and more recently in the setting of solid forms of cancer, including melanoma (Perica et al., 2015). Since a major concern with developing a combination therapy integrating chemotherapeutic drugs (genotoxic, HSP90i) reflects possible negative impact of this drug class on the patient's endogenous immune system. To avoid this, we hypothesized that one should apply lower doses of anti-cancer drugs in concert with autologous ACT using CD8^{+} T cells primed against DNA-RP-derived epitopes.

The hypothesis for this study was that HSP90i can conditionally sensitize melanoma cells to the tumoricidal action of chemotherapy as well as $CD8^+$ T cells reactive against MHC class I-presented peptides derived from tumor cell overexpressed DNA-RP (intrinsic or after treatment with TMZ) that constitute HSP90 client proteins. Our belief was that HSP90i could conditionally (and transiently) upregulate the stoichiometry of a pool of HSP90 client protein-derived peptides presented in the global "repertoire" of MHC class I/peptide complexes presented on the tumor cell surface, allowing for improved recognition and cytotoxicity mediated by specific $CD8^+$ T effector cells. No studies have thus far focused on the ability of HSP90i to conditionally increase the degradation and subsequent presentation of DNA-RP-derived peptides in MHC class I complexes for recognition by the immune system *in vivo*. The results of our work have potential to shape the design and implementation of novel combination chemoimmunotherapies for a broad range of patients with cancer, including those afflicted with melanoma.

We wanted to first characterize the expression of DNA repair client proteins by melanoma cells and determine the anti-melanoma efficacy of combined chemotherapy +/- HSP90i. We chose Temozolomide (TMZ) as a preferred chemotherapy drug as it is commonly used for both melanoma and glioblastoma, with a known capacity to cross the blood-brain barrier (Quirt et al., 2007), making it theoretically useful in the context of treating melanoma metastases that commonly traffic to the brain, where they are fatal. Since STA9090 has shown promising results when applied in combination with Docetaxel *in vivo* (Ying et al., 2012) and is actively being evaluated in the clinic, this 2^{nd} generation drug was an obvious choice as the preferred HSP90i for this translational research.

Our initial studies reinforced the inability of TMZ alone to promote melanoma apoptosis, but that when combined with the HSP90i STA9090, tumor cell sensitization to TMZ-induced apoptosis was increased by a factor of 5.

From studies performed by the Kauffmann group, it was evident that DNA-RP become overexpressed during the transition from primary-to-metastatic melanoma, in association with melanoma resistance to conventional chemo- and radiotherapies (Kauffmann et al., 2008). In order to identify the cell cycle phase involved in melanoma resistance to TMZ, we investigated cell cycle transition in the absence or presence of TMZ +/- STA9090. Our results showed that TMZ induces enhanced G2/M arrest that may be reversed by cotreatment with STA9090. Based on this finding, we selected for further analysis several key proteins involved in the HR and NHEJ repair pathways that are HSP90 clients. Amongst the considered DNA-RP, ATR, PRKDC, MRE11, NBN, RAD50 and RAD51 were identified as overexpressed melanoma proteins in the Kauffmann study. To ensure that these proteins were in fact overexpressed in the specific melanoma cell lines that we selected for modeling purposes (i.e., murine melanomas B16 and BPR; human metastatic melanoma cell lines, Mel526 and Mel624), we analyzed lysates of the cultured melanoma cell lines using SDS-PAGE and Western Blotting for specific DNA-RP expression levels. We detected all of these DNA-RP in each of the melanoma cell lines, with the exception of PRKDC in Mel526 and RAD51 in Mel624.

Furthermore, we noted that TMZ promoted increased expression of DNA-RP (i.e., RAD50, BRCA2, NBN, ATR) and HSP90 proteins in melanoma cells *in vitro* and *in vivo.* Subsequent quantitative RT-PCR analysis suggested that while tumor cell transcription of HSP90 was increased after TMZ treatment, levels of mRNA encoding DNA-RP were not augmented (with the possible exception of PRKDC) by drug treatment. This result is consistent

with a mechanism in which TMZ-induced transcriptional activation of HSP90 leads to enhanced post-translational stabilization and accumulation of DNA-RP client proteins in treated tumor cells.

This suggested that DNA-RP overexpression in TMZ-treated melanomas could be antagonized by HSP90i. To determine the effect HSP90i DNA repair protein expression levels in this setting, we cultured melanoma cells in the presence of HSP90i NVP-AUY922 and STA9090 after the cells had been preconditioned with TMZ. Based on Western Blotting analyses, we determined that both HSP90i promoted the degradation of DNA-RPs. In extended studies, we also assessed melanoma expression of the DNA-RP RAD51 after radiation. Similar to the impact of TMZ, we observed increased RAD51 protein expression after radiation, with the accumulated protein then degraded upon subsequent treatment of the tumor cells with HSP90i NVP-AUY922.

From an immunologic perspective, the degradation of these DNA-RP is of interest since this process is mediated in the tumor cytosol by the 26S proteasome, a multi-catalytic machine responsible for generating small peptides that ultimately become "presented" in MHC class I complexes on the tumor cell surface as "barcodes" for antigen-specific $CD8^+$ T cell recognition. To confirm this operational paradigm, we treated the murine B16 and human Mel526 melanoma cell lines *in vitro* with TMZ +/- STA9090 +/- MG132 (a proteasome inhibitor). Although this system has not yet been optimized, we observed the trend that STA9090-induced degradation of DNA-RP previously upregulated in melanoma cells by TMZ was at least partially prevented by MG132. It is important to note that these cell lines are polyclonal and exhibit genetic and phenotypic heterogeneity, hence it might be expected that not all proteins under analysis behaved in a concerted manner. For example, in B16 melanoma cells, RAD50 was not overexpressed when treated with TMZ, NBN was not protected from HSP90i-induced degradation by MG132.

In human Mel526 cells, expression of MRE11 protein was upregulated by TMZ, but not subsequently induced to degrade by STA9090 treatment. Such variability in the fate of individual DNA-RP in the face of these drug treatments reinforces the need to consider the broader impact of the combination therapy on the general class of DNA-RP proteins that represent HSP90 clients across a heterogeneous population of tumor cells.

Since HSP90i promoted the degradation of several of the identified DNA-RP in a proteasome-dependent manner, we next wanted to investigate the ramifications of such treatments on tumor cell recognition by DNA-RP-specific $CD8^+$ T cells. Since $CD8^+$ T cell epitopes in these proteins had not been previously reported in the literature, we used peptide binding algorithms to identify candidate epitopes with highest predicted binding affinity score for the two forms of MHC class I molecules expressed in C57BL/6 mice (i.e., H-$2K^b$ and H-$2D^b$). This led to the synthesis of 30 peptides that were then segregated in three pools of 10 peptides each that were used to vaccinate and boost naïve mice. After three rounds of immunization, mice were euthanized and splenic $CD8^+$ T cells analyzed for their ability to produce the effector cytokine IFN-γ (quantitated by ELISA) in response to specific peptide restimulation *in vitro*. From the results of three such experiments, we selected nine DNA-RP-derived peptides that were most immunogenic (i.e., $RAD51_{58\text{-}66}$, $RAD51_{212\text{-}219}$ $RAD50_{1180\text{-}1187,}$ $RAD50_{391\text{-}399,}$ $ATR_{1390\text{-}1397,}$ $PRKDC_{620\text{-}628,}$ $MRE11_{109\text{-}116}$, $MRE11_{208\text{-}216}$ $NBN_{273\text{-}281}$) for use in combination chemoimmunotherapy studies.

We determined that $CD8^+$ T cells isolated from mice vaccinated against a pool of these nine peptides exhibited increased recognition of B16 cells that had been pre-treated with TMZ + HSP90i (versus those treated with either single drug or left untreated). These data suggest that even though B16 melanoma cells are resistant to TMZ, the ability of TMZ to augment DNA-RP

expression and of HSP90i to convert this overexpressed protein into immunogenic peptides that allow for improved recognition by DNA-RP vaccine-induced $CD8^+$ T cells. The ability to generate such anti-tumor effector T cells via vaccination also provided us with the opportunity to investigate combination chemoimmunotherapy integrating TMZ, HSP90i and DNA-RP-specific ACT.

As discussed in Chapter 3, we observed that *in vivo* treatment of established B16 melanoma in C57BL/6 mice with TMZ did not have beneficial effects with regard to the rate of tumor progression. As a consequence, we retained TMZ as a baseline treatment for all four combination therapy groups in studies discussed in Chapter 4. After initial treatment of the mice with TMZ (to upregulate DNA-RP expression in tumor cells), the animals were randomized, with individual groups of mice then treated with: i.) two weekly i.p. doses of STA9090, ii.) two weekly doses of i.v. ACT, iii.) two weekly doses of STA9090 (i.p.) and ACT (i.v.), or iv.) PBS (i.p.). We observed that melanomas in the group treated with combined TMZ/HSP90i + ACT grew more slowly than comparable tumors in mice in any other treatment cohort.

To further validate the findings from our laboratory that HSP90i enhances T cell infiltration into tumor lesions *in vivo* (Rao et al., 2012), single-cell suspensions from recovered spleens and tumor digests were analyzed by flow data, focusing on $CD8^+$ T cell content. We noted that the combination TMZ/HSP90i + ACT treatment group contained the highest level of $CD8^+$ T cell infiltration in tumors. This finding was also confirmed in immunofluorescence microscopy analyses of tumor sections based on a $CD3^+Tbet^+$ T cell phenotype (consistent with the presence of Type-1 inflammatory/cytotoxic T cells). Additional studies were performed in which we pre-labeled the anti-DNA-RP $CD8^+$ T cells prior to injection into tumor-bearing mice with a deep red dye. Subsequent analysis of harvested spleens and tumors revealed no

meaningful difference in the frequency of labeled cells in the spleens of treated mice (TMZ + ACT vs. TMZ/HSP90i + ACT), while we found that the combination TMZ/HSP90i + ACT had over 30-fold more labeled cells in their tumors when compared to the tumors from mice treated with TMZ + ACT only. These findings support superior recruitment of therapeutic ACT T cells into the tumors of mice receiving TMZ/HSP90i + ACT-based therapy.

Although this study was performed solely in melanoma models, we predict that the preferred combination chemoimmunotherapy that we have identified will prove efficacious against other forms of cancer as well. This study also provides a rationale for the use of HSP90i STA9090 at a dose (25 mg/kg) far below the MTD in order to enhance therapeutic T cell infiltration into tumors, in association with superior anti-tumor efficacy, particularly when used in combination with ACT-based immunotherapy approaches. The use of low dose, metronomic protocols for administering HSP90i is likely to optimize overall clinical efficacy based on a balance between the intrinsic anti-tumor effects of this drug class and its impact on effector vs. regulatory immunity in the tumor microenvironment. Future studies will need to refine the dose and schedule for optimal therapeutic benefits associated with HSP90i-based (combination) therapies.

A study by Proia and Kaufmann suggests that the combination of weekly-administered STA9090 (125 mg/kg) with anti-PD-L1 antibody STI-A1015 promotes superior antitumor activity *in vivo* when compared with either single agent, with similar results was observed in pre-clinical B16 melanoma models (Proia & Kaufmann, 2015). Considering the current success of immune checkpoint blockade protocols using PD-1 and/or PD-L1 targeted therapies in melanoma, it is both logical and important to further analyze: i) how our combination chemoimmunotherapy impacts expression of PD-L1 within the TME, and ii.) how this regimen

might be further improved in its anti-tumor efficacy by further combination with anti-PD-1 and/or anti-PD-L1 antagonist antibodies. As discussed in Chapter 4, combination with effective HSP70i may also represent another alternative to HSP90i or they can be used therapeutically in combination to minimize compensatory mechanisms of adaptive tumor resistance *in vivo*.

The results of our studies may be particularly beneficial to patients who have undergone chemotherapy in the past and have developed treatment-associated resistance and disease progression. Although our experiments have focused on use of TMZ as a chemotherapeutic agent to conditionally upregulate DNA-RP expression in tumors, we also noted similar results for the commonly-prescribed anthracycline agent, Doxorubicin. This may expand the generality of our findings and assist in the future development of novel combination chemoimmunotherapy approaches that will have a significant public health impact.

APPENDIX – ABBREVIATIONS

17- AAG, 17-N-allylamino-17-demethoxygelandamycin

17- DMAG, 17- Dimethylaminoethylamino-17-demethoxygelandamycin

Abs, Antibodies

ACT, Adoptive Cell Therapy

Ad-IL12, Adenovirus induced Interleukin 12

ADP, Adenosine diphosphate

AHA1, Activator of Heat Shock Protein ATPase 1

AIF, Apoptosis-Inducing Factor

AKT1, Also known as PKB – Protein Kinase B

ANOVA, Analysis of Variance

APC, Antigen Presenting Cells

ASK1, Apoptosis Signal-Regulating Kinase 1

ATCC, American Type Culture Collection

ATM. Ataxia Telangiectasia Mutated serine-protein kinase

ATP, Adenosine Triphosphate

ATR, Ataxia Telangiectasia and Rad3 related protein kinase

BER, Base Excision Repair

BRAF, B-Raf proto-oncogene

BRCA1, breast cancer 1

BRCA2, breast cancer 2

CAR, Chimeric Antigen Receptor

CD, Cluster of Differentiation

CDC37, Cell Division Cycle 37

CHEK1, Checkpoint Kinase 1

CHEK2, Checkpoint Kinase 2

CM, Complete Media

CML, Chronic Myeloid Leukemia

CNS, Central Nervous System

CRT, Chemo Radio Therapy

CT, Chemotherapy

CTL, Cytotoxic T Lymphocyte

CTAG1B, a cancer testis antigen

CTLA4, Cytotoxic T Lymphocyte Antigen 4

CYP40, Cyclophilin 40

CYPD, Cyclophilin D

DAB, Dabrafenib

DAPI, 4',6 –Diamidino – 2- Phenylindole

DC, Dendritic Cells

DMSO – Dimethyl Sulfoxide

DNA, DeoxyriboNucleic Acid

DNA-RP, DNA Repair Protein

DOX, Doxorubicin

DSB, Double Strand Break

ER, Endoplasmic Reticulum

ERBB2, Also known as Her2/Neu

ERCC, Excision Repair Cross-Completion

ERK, Extracellular Signal-Regulated Kinase

FANCD2, Fanconi Anemia Completion Group D2

FDA, Food & Drug Administration

Fmoc, Fluorenylmethyloxycarbonyl chloride

GA, Geldanamycin

GBM, Glioblastoma

Gp100, Glycoprotein 100

GRP, Glucose Regulated Protein

Gy, Gray (unit of radiation)

H, Hours

H2AX, H2A histone family member X

H-2Db/ H-2Kb, Murine class I molecules in the H-2 haplotype mouse

HDACi, Histone deacetylase inhibitor

Her2, Human Epidermal Growth Factor Receptor 2, aka ERBB2

HIP, Carboxyl-terminus of HSP70 interacting protein

HLA, Human Leukocyte Antigen

HOP, HSP70/HSP90 Organizing Protein

Hprt, Hypoxanthine Phosphoribosyltransferase

HR, Homologous Recombination

HRP, Horeradish Peroxidase

HSF1, Heat Shock Factor Protein 1

HSP, Heat Shock Protein

HSP90i, HSP90 inhibitor

i.p., Intraperitoneal

i.v., Intravenous

IACUC, Institutional Animal Care and Use Committee

IARC, International Agency for Research Cancer

IL, Interleukin

IR, Irradiation

KIT, KIT Proto-Oncogene Receptor Tyrosine Kinase

MAPK, Mitogen-Activated Protein Kinase

MDSC, Myeloid-Derived Suppressor Cell

MEK, alias for MAP2K (Mitogen Activated Protein Kinase Kinase)

MHC, Major Histocompatibility complex

MMR, Mismatch Repair

MRE11, Meiotic Recombination 11

MRN, complex of MRE11, RAD50, & Nibrin

MSH2, Muts Homolog 2

NBN, Nibrin

NER, Nucleotide Excision Repair

NHEJ, Non-Homologous End Joining

NK, Natural Killer Cells

NMA, Non-Myeloablative

NSCLC, Non-Small Cell Lung Cancer

NVP-AUY922, an HSP90 inhibitor also known as Luminespib

ORR, Objective Rate Response

PBMC, Peripheral Blood Mononuclear Cell

PCNA, Proliferating Cell Nuclear Antigen

PD-1, Programmed Cell Death 1 receptor

PDGFRs, Platelet-derived Growth Factor Receptors

PD-L1, Programmed Cell Death 1 receptor Ligand 1

PI, Propidium Iodide

PI3K, Phosphoinositide 3-kinase

PMEL, Premelanosome protein

PVDF, Polyvinylidene flluoride

RAF, Raf-1 proto-oncogene, serine/threonine kinase

RAS, Viral Oncogene Homolog family of related proteins

REP, Rapid Expansion Protocol

rmGM-CSF, Recombinant Murine Granulocyte/Macrophage Colony-Stimulating Factor

ROS, Reactive Oxygen Species

RPA, Replication Protein A

s.c., Subcutaneous

SSB, Single Strand Break

STA9090, Small molecule HSP90 inhibitor also known as Ganetespib

TAM, Tumor-Associated Macrophages

TAP, Transporter Associated with antigen processing

T-bet, Tbox Transcription factor

Tc1, Type-1 $CD8^+$ T

TCR, T-Cell Receptor

Teff, T effector cells

TIL, Tumor-Infiltrating Lymphocyte

TME, Tumor Microenvironment

TMZ, Temozolomide

TNF, Tumor Necrosis Factor

TP53, tumor (suppressor) protein p53

TPR, Translocated Promoter Region

TRAP1, Tumor necrosis factor Receptor-Associated Protein 1

Treg, Regulatory T cells

TUNEL, Terminal deoxnucleotidyl transferase dUTP Nick End Labeling

US, United States

UT, Untreated

UV, Ultra Violet

VEGFRs, Vascular Endothelial Growth Factor Receptors

XRCC4, X-ray Repair Cross-Complementing protein 4

BIBLIOGRAPHY

Acquaviva, J., Smith, D. L., Jimenez, J.-P., Zhang, C., Sequeira, M., He, S., … Proia, D. A. (2014). Overcoming Acquired BRAF Inhibitor Resistance in Melanoma via Targeted Inhibition of Hsp90 with Ganetespib. *Molecular Cancer Therapeutics*, *13*(2), 353–363. doi:10.1158/1535-7163.MCT-13-0481

Alvino, E., Castiglia, D., Caporali, S., Pepponi, R., Caporaso, P., Lacal, P. M., … D'Atri, S. (2006). A single cycle of treatment with temozolomide, alone or combined with O(6)-benzylguanine, induces strong chemoresistance in melanoma cell clones in vitro: role of O(6)-methylguanine-DNA methyltransferase and the mismatch repair system. *International Journal of Oncology*, *29*(4), 785–97. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16964376

Bae, J., Munshi, A., Li, C., Samur, M., Prabhala, R., Mitsiades, C., … Munshi, N. C. (2013). Heat shock protein 90 is critical for regulation of phenotype and functional activity of human T lymphocytes and NK cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *190*(3), 1360–71. doi:10.4049/jimmunol.1200593

Bali, P., Pranpat, M., Bradner, J., Balasis, M., Fiskus, W., Guo, F., … Bhalla, K. (2005). Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. *The Journal of Biological Chemistry*, *280*(29), 26729–34. doi:10.1074/jbc.C500186200

Barrott, J. J., & Haystead, T. A. J. (2013). Hsp90, an unlikely ally in the war on cancer. *The FEBS Journal*, *280*(6), 1381–96. doi:10.1111/febs.12147

Bauer, S., Yu, L. K., Demetri, G. D., & Fletcher, J. A. (2006). Heat shock protein 90 inhibition in imatinib-resistant gastrointestinal stromal tumor. *Cancer Research*, *66*(18), 9153–61. doi:10.1158/0008-5472.CAN-06-0165

Becker, B., Multhoff, G., Farkas, B., Wild, P.-J., Landthaler, M., Stolz, W., & Vogt, T. (2004). Induction of Hsp90 protein expression in malignant melanomas and melanoma metastases. *Experimental Dermatology*, *13*(1), 27–32. doi:10.1111/j.0906-6705.2004.00114.x

Berghoff, A. S., Fuchs, E., Ricken, G., Mlecnik, B., Bindea, G., Spanberger, T., Preusser, M. (2015). Density of tumor-infiltrating lymphocytes correlates with extent of brain edema and overall survival time in patients with brain metastases. *Oncoimmunology*, *5*(1), e1057388. doi:10.1080/2162402X.2015.1057388

Besser, M. J., Shapira-Frommer, R., Treves, A. J., Zippel, D., Itzhaki, O., Hershkovitz, L., … Schachter, J. (2010). Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *16*(9), 2646–55. doi:10.1158/1078-0432.CCR-10-0041

Bhatia, S., Tykodi, S. S., & Thompson, J. A. (2009). Treatment of metastatic melanoma: an overview. *Oncology (Williston Park, N.Y.)*, *23*(6), 488–96. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19544689

Binder, R. J., & Srivastava, P. K. (2005). Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells. *Nature Immunology*, *6*(6), 593–599. doi:10.1038/ni1201

Blagosklonny, M. V. (2002). Hsp-90-associated oncoproteins: multiple targets of geldanamycin and its analogs. *Leukemia*, *16*(4), 455–62. doi:10.1038/sj.leu.2402415

Blagosklonny, M. V, Toretsky, J., Bohen, S., & Neckers, L. (1996). Mutant conformation of p53 translated in vitro or in vivo requires functional HSP90. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(16), 8379–83. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8710879

Brahmer, J. R., Drake, C. G., Wollner, I., Powderly, J. D., Picus, J., Sharfman, W. H., … Topalian, S. L. (2010). Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *28*(19), 3167–75. doi:10.1200/JCO.2009.26.7609

Brahmer, J. R., Tykodi, S. S., Chow, L. Q. M., Hwu, W.-J., Topalian, S. L., Hwu, P., … Wigginton, J. M. (2012). Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. *New England Journal of Medicine*, *366*(26), 2455–2465. doi:10.1056/NEJMoa1200694

Buchner, J., Welch, W. J., Feramisco, J. R., Borkovich, K. A., al., et, Cutforth, T., … Lindquist, S. (1999). Hsp90 & Co. – a holding for folding. *Trends in Biochemical Sciences*, *24*(4), 136–141. doi:10.1016/S0968-0004(99)01373-0

Butler, L. M., Ferraldeschi, R., Armstrong, H. K., Centenera, M. M., & Workman, P. (2015). Maximizing the Therapeutic Potential of HSP90 Inhibitors. *Molecular Cancer Research : MCR*, *13*(11), 1445–51. doi:10.1158/1541-7786.MCR-15-0234

Cancer Facts and Figures 2016. (2016). Atlanta: American Cancer Society.

Cavanaugh, A., Juengst, B., Sheridan, K., Danella, J. F., & Williams, H. (2015). Combined inhibition of heat shock proteins 90 and 70 leads to simultaneous degradation of the oncogenic signaling proteins involved in muscle invasive bladder cancer. *Oncotarget*, *6*(37), 39821–38. doi:10.18632/oncotarget.5496

Chabner, B. A., & Roberts, T. G. (2005). Timeline: Chemotherapy and the war on cancer. *Nature Reviews Cancer*, *5*(1), 65–72. doi:10.1038/nrc1529

Chapman, P. B., Hauschild, A., Robert, C., Haanen, J. B., Ascierto, P., Larkin, J., … BRIM-3 Study Group. (2011). Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *The New England Journal of Medicine*, *364*(26), 2507–16. doi:10.1056/NEJMoa1103782

Che, Y., Best, O. G., Zhong, L., Kaufman, K. L., Mactier, S., Raftery, M., … Christopherson, R. I. (2013). Hsp90 Inhibitor SNX-7081 Dysregulates Proteins Involved with DNA Repair and Replication and the Cell Cycle in Human Chronic Lymphocytic Leukemia (CLL) Cells. *Journal of Proteome Research*, *12*(4), 1710–22. doi:10.1021/pr301055y

Choi, E. J., Cho, B. J., Lee, D. J., Hwang, Y. H., Chun, S. H., Kim, H. H., & Kim, I. A. (2014). Enhanced cytotoxic effect of radiation and temozolomide in malignant glioma cells: targeting PI3K-AKT-mTOR signaling, HSP90 and histone deacetylases. *BMC Cancer*, *14*(1), 17. doi:10.1186/1471-2407-14-17

Ciocca, D. R., Arrigo, A. P., & Calderwood, S. K. (2013). Heat shock proteins and heat shock factor 1 in carcinogenesis and tumor development: an update. *Archives of Toxicology*, *87*(1), 19–48. doi:10.1007/s00204-012-0918-z

Cohen, C. J., Zheng, Z., Bray, R., Zhao, Y., Sherman, L. A., Rosenberg, S. A., & Morgan, R. A. (2005). Recognition of fresh human tumor by human peripheral blood lymphocytes transduced with a bicistronic retroviral vector encoding a murine anti-p53 TCR. *Journal of Immunology (Baltimore, Md. : 1950)*, *175*(9), 5799–808. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16237072

Cohen, J., & Sznol, M. (2015). Therapeutic combinations of immune-modulating antibodies in melanoma and beyond. *Seminars in Oncology*, *42*(3), 488–94. doi:10.1053/j.seminoncol.2015.02.014

Cooper, Z. A., Juneja, V. R., Sage, P. T., Frederick, D. T., Piris, A., Mitra, D., … Wargo, J. A. (2014). Response to BRAF Inhibition in Melanoma Is Enhanced When Combined with Immune Checkpoint Blockade. *Cancer Immunology Research*, *2*(7), 643–654. doi:10.1158/2326-6066.CIR-13-0215

Csermely, P., Schnaider, T., Soti, C., Prohászka, Z., & Nardai, G. (1998). The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacology & Therapeutics*, *79*(2), 129–68. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9749880

Dai, H., Wang, Y., Lu, X., & Han, W. (2016). Chimeric Antigen Receptors Modified T-Cells for Cancer Therapy. *Journal of the National Cancer Institute*, *108*(7), djv439. doi:10.1093/jnci/djv439

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., … Futreal, P. A. (2002). Mutations of the BRAF gene in human cancer. *Nature*, *417*(6892), 949–954. doi:10.1038/nature00766

Dexheimer, T. S. (2013). DNA Repair Pathways and Mechanisms. In *DNA Repair of Cancer Stem Cells* (pp. 19–32). Dordrecht: Springer Netherlands. doi:10.1007/978-94-007-4590-2_2

Dietlein, F., Thelen, L., Reinhardt, H. C., Reinhardt, H. C., Yaffe, M. B., Jackson, S. P., … al., et. (2014). Cancer-specific defects in DNA repair pathways as targets for personalized therapeutic approaches. *Trends in Genetics*, *30*(8), 326–339. doi:10.1016/j.tig.2014.06.003

Dote, H., Burgan, W. E., Camphausen, K., & Tofilon, P. J. (2006). Inhibition of hsp90 compromises the DNA damage response to radiation. *Cancer Research*, *66*(18), 9211–20. doi:10.1158/0008-5472.CAN-06-2181

Dudley, M. E., Gross, C. A., Langhan, M. M., Garcia, M. R., Sherry, R. M., Yang, J. C., … Rosenberg, S. A. (2010). CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *16*(24), 6122–31. doi:10.1158/1078-0432.CCR-10-1297

Dudley, M. E., Yang, J. C., Sherry, R., Hughes, M. S., Royal, R., Kammula, U., … Rosenberg, S. A. (2008). Adoptive Cell Therapy for Patients With Metastatic Melanoma: Evaluation of Intensive Myeloablative Chemoradiation Preparative Regimens. *Journal of Clinical Oncology*, *26*(32), 5233–5239. doi:10.1200/JCO.2008.16.5449

Elliott, T. (2006). The “chop-and-change” of MHC class I assembly. *Nature Immunology*, *7*(1), 7–9. doi:10.1038/ni0106-7

Falchook, G. S., Long, G. V, Kurzrock, R., Kim, K. B., Arkenau, T. H., Brown, M. P., … Rosen, N. (2012). Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumours: a phase 1 dose-escalation trial. *The Lancet*, *379*(9829), 1893–1901. doi:10.1016/S0140-6736(12)60398-5

FDA Approval for Temozolomide - National Cancer Institute. (n.d.). Retrieved from http://www.cancer.gov/about-cancer/treatment/drugs/fda-temozolomide

Finn, O. J. (2008). Cancer Immunology. *New England Journal of Medicine*, *358*(25), 2704–2715. doi:10.1056/NEJMra072739

Fojo, T. (2001). Cancer, DNA Repair Mechanisms, and Resistance to Chemotherapy. *JNCI Journal of the National Cancer Institute*, *93*(19), 1434–1436. doi:10.1093/jnci/93.19.1434

Fulda, S., Galluzzi, L., & Kroemer, G. (2010). Targeting mitochondria for cancer therapy. *Nature Reviews Drug Discovery*, *9*(6), 447–464. doi:10.1038/nrd3137

Gilboa, E. (2001). The risk of autoimmunity associated with tumor immunotherapy. *Nature Immunology*, *2*(9), 789–792. doi:10.1038/ni0901-789

Goetz, M. P., Toft, D. O., Ames, M. M., & Erlichman, C. (2003). The Hsp90 chaperone complex as a novel target for cancer therapy. *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO*, *14*(8), 1169–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12881371

Goldberg, A. L., Cascio, P., Saric, T., & Rock, K. L. (2002). The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. *Molecular Immunology*, *39*(3-4), 147–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12200047

Guida, M., Pisconti, S., Pisconte, S., & Colucci, G. (2012). Metastatic melanoma: the new era of targeted therapy. *Expert Opinion on Therapeutic Targets*, *16 Suppl 2*, S61–70. doi:10.1517/14728222.2011.645807

Ha, K., Fiskus, W., Rao, R., Balusu, R., Venkannagari, S., Nalabothula, N. R., & Bhalla, K. N. (2011). Hsp90 inhibitor-mediated disruption of chaperone association of ATR with hsp90 sensitizes cancer cells to DNA damage. *Molecular Cancer Therapeutics*, *10*(7), 1194–206. doi:10.1158/1535-7163.MCT-11-0094

Haggerty, T. J., Dunn, I. S., Rose, L. B., Newton, E. E., Pandolfi, F., & Kurnick, J. T. (2014). Heat shock protein-90 inhibitors enhance antigen expression on melanomas and increase T cell recognition of tumor cells. *PloS One*, *9*(12), e114506. doi:10.1371/journal.pone.0114506

Hanahan, D., & Weinberg, R. A. (2000). Hallmarks of cancer. *Cell*, *100*(1), 57-70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10647931

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646–674. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21376230

Harris, T. J., Drake, C. G., Dunn, G., Bruce, A., Ikeda, H., Old, L., … June, C. (2013). Primer on tumor immunology and cancer immunotherapy. *Journal for ImmunoTherapy of Cancer*, *1*(1), 12. doi:10.1186/2051-1426-1-12

He, S., Smith, D. L., Sequeira, M., Sang, J., Bates, R. C., & Proia, D. A. (2014). The HSP90 inhibitor ganetespib has chemosensitizer and radiosensitizer activity in colorectal cancer. *Investigational New Drugs*, *32*(4), 577–86. doi:10.1007/s10637-014-0095-4

Heath, W. R., Belz, G. T., Behrens, G. M. N., Smith, C. M., Forehan, S. P., Parish, I. A., … Villadangos, J. A. (2004). Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunological Reviews*, *199*, 9–26. doi:10.1111/j.0105-2896.2004.00142.x

Helleday, T., Petermann, E., Lundin, C., Hodgson, B., & Sharma, R. A. (2008). DNA repair pathways as targets for cancer therapy. *Nature Reviews Cancer*, *8*(3), 193–204. doi:10.1038/nrc2342

Hickey, E., Brandon, S. E., Sadis, S., Smale, G., & Weber, L. A. (1986). Molecular cloning of sequences encoding the human heat-shock proteins and their expression during hyperthermia. *Gene*, *43*(1-2), 147–54. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3019832

Hinz, J. M., Yamada, N. A., Salazar, E. P., Tebbs, R. S., & Thompson, L. H. (2005). Influence of double-strand-break repair pathways on radiosensitivity throughout the cell cycle in CHO cells. *DNA Repair*, *4*, 782–792. doi:10.1016/j.dnarep.2005.03.005

Hodi, F. S., O'Day, S. J., McDermott, D. F., Weber, R. W., Sosman, J. A., Haanen, J. B., … Urba, W. J. (2010). Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. *New England Journal of Medicine*, *363*(8), 711–723. doi:10.1056/NEJMoa1003466

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature*, *411*(6835), 366–74. doi:10.1038/35077232

Hoeijmakers, J. H. J. (2009). DNA damage, aging, and cancer. *The New England Journal of Medicine*, *361*(15), 1475–85. doi:10.1056/NEJMra0804615

Imai, T., Kato, Y., Kajiwara, C., Mizukami, S., Ishige, I., Ichiyanagi, T., … Diane Mathis, by. (n.d.). Heat shock protein 90 (HSP90) contributes to cytosolic translocation of extracellular antigen for cross-presentation by dendritic cells Laboratories for. doi:10.1073/pnas.1108372108

Jewell, R., Conway, C., Mitra, A., Randerson-Moor, J., Lobo, S., Nsengimana, J., … Newton-Bishop, J. (2010). Patterns of expression of DNA repair genes and relapse from melanoma. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *16*(21), 5211–21. doi:10.1158/1078-0432.CCR-10-1521

Jhaveri, K., Chandarlapaty, S., Lake, D., Gilewski, T., Robson, M., Goldfarb, S., … Modi, S. (2014). A phase II open-label study of ganetespib, a novel heat shock protein 90 inhibitor for patients with metastatic breast cancer. *Clinical Breast Cancer*, *14*(3), 154–60. doi:10.1016/j.clbc.2013.12.012

Johnson, D. B., Sosman, J. A., H, D., T, E., RD, C., FS, H., I, P. (2015). Therapeutic Advances and Treatment Options in Metastatic Melanoma. *JAMA Oncology*, *1*(3), 380. doi:10.1001/jamaoncol.2015.0565

Johnson, L. A., Heemskerk, B., Powell, D. J., Cohen, C. J., Morgan, R. A., Dudley, M. E., … Rosenberg, S. A. (2006). Gene transfer of tumor-reactive TCR confers both high avidity and tumor reactivity to nonreactive peripheral blood mononuclear cells and tumor-infiltrating lymphocytes. *Journal of Immunology (Baltimore, Md. : 1950)*, *177*(9), 6548–59. Retrieved from

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2174608&tool=pmcentrez&rendertype=abstract

Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., & Burrows, F. J. (2003). A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature*, *425*(6956), 407–10. doi:10.1038/nature01913

Kastan, M. B., & Bartek, J. (2004). Cell-cycle checkpoints and cancer. *Nature*, *432*(7015), 316–323. doi:10.1038/nature03097

Kauffmann, A., Rosselli, F., Lazar, V., Winnepenninckx, V., Mansuet-Lupo, A., Dessen, P., … Sarasin, A. (2008). High expression of DNA repair pathways is associated with metastasis in melanoma patients. *Oncogene*, *27*(5), 565–73. doi:10.1038/sj.onc.1210700

Kawabe, M., Mandic, M., Taylor, J. L., Vasquez, C. A., Wesa, A. K., Neckers, L. M., & Storkus, W. J. (2009). Heat shock protein 90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin enhances EphA2+ tumor cell recognition by specific CD8+ T cells. *Cancer Research*, *69*(17), 6995–7003. doi:10.1158/0008-5472.CAN-08-4511

Kawakami, Y., Robbins, P. F., & Rosenberg, S. A. (1996). Human melanoma antigens recognized by T lymphocytes. *The Keio Journal of Medicine*, *45*(2), 100–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8683899

Kekatpure, V. D., Dannenberg, A. J., & Subbaramaiah, K. (2009). HDAC6 modulates Hsp90 chaperone activity and regulates activation of aryl hydrocarbon receptor signaling. *The Journal of Biological Chemistry*, *284*(12), 7436–45. doi:10.1074/jbc.M808999200

Kelley, M. R., Logsdon, D., & Fishel, M. L. (n.d.). Targeting DNA repair pathways for cancer treatment: what's new? doi:10.2217/fon.14.60

Khalil, D. N., Smith, E. L., Brentjens, R. J., & Wolchok, J. D. (2016). The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nature Reviews Clinical Oncology*, *13*(5), 273–290. doi:10.1038/nrclinonc.2016.25

Khan, M. K., Khan, N., Almasan, A., & Macklis, R. (2011). Future of radiation therapy for malignant melanoma in an era of newer, more effective biological agents. *OncoTargets and Therapy*, *4*, 137–48. doi:10.2147/OTT.S20257

Khanna, K. K., & Jackson, S. P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. *Nature Genetics*, *27*(3), 247–254. doi:10.1038/85798

Kinsella, T. J. (2009). Understanding DNA damage response and DNA repair pathways: applications to more targeted cancer therapeutics. *Seminars in Oncology*, *36*(2 Suppl 1), S42–51. doi:10.1053/j.seminoncol.2009.02.004

Ko, J.-C., Chen, H.-J., Huang, Y.-C., Tseng, S.-C., Weng, S.-H., Wo, T.-Y., … Lin, Y.-W. (2012). HSP90 inhibition induces cytotoxicity via down-regulation of Rad51 expression and DNA repair capacity in non-small cell lung cancer cells. *Regulatory Toxicology and Pharmacology : RTP*, *64*(3), 415–24. doi:10.1016/j.yrtph.2012.10.003

Koga, F., Kihara, K., & Neckers, L. (2009). Inhibition of Cancer Invasion and Metastasis by Targeting the Molecular Chaperone Heat-shock Protein 90. *Anticancer Res*, *29*(3), 797–807. Retrieved from http://ar.iiarjournals.org/content/29/3/797.long

Kong, Y. M., Jacob, J. B., Flynn, J. C., Elliott, B. E., & Wei, W.-Z. (2009). Autoimmune thyroiditis as an indicator of autoimmune sequelae during cancer immunotherapy. *Autoimmunity Reviews*, *9*(1), 28–33. doi:10.1016/j.autrev.2009.02.034

Lampen, M. H., & van Hall, T. (2011). Strategies to counteract MHC-I defects in tumors. *Current Opinion in Immunology*, *23*(2), 293–8. doi:10.1016/j.coi.2010.12.005

Leone, P., Shin, E.-C., Perosa, F., Vacca, A., Dammacco, F., & Racanelli, V. (2013). MHC class I antigen processing and presenting machinery: organization, function, and defects in tumor cells. *Journal of the National Cancer Institute*, *105*(16), 1172–87. doi:10.1093/jnci/djt184

Li, Z., & Srivastava, P. (2004). Heat-shock proteins. *Current Protocols in Immunology / Edited by John E. Coligan ... [et Al.]*, *Appendix 1*, Appendix 1T. doi:10.1002/0471142735.ima01ts58

Liao, Y., Ou, J., Deng, J., Geng, P., Zeng, R., Tian, Y., … Ruan, Z. (2013). Clinical implications of the tumor-infiltrating lymphocyte subsets in colorectal cancer. *Medical Oncology*, *30*(4), 727. doi:10.1007/s12032-013-0727-0

Lin, C.-C., Tu, C.-F., Yen, M.-C., Chen, M.-C., Hsieh, W.-J., Chang, W.-C., … Lai, M.-D. (2007). Inhibitor of heat-shock protein 90 enhances the antitumor effect of DNA vaccine targeting clients of heat-shock protein. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *15*(2), 404–10. doi:10.1038/sj.mt.6300014

Lu, X., Xiao, L., Wang, L., & Ruden, D. M. (2012). Hsp90 inhibitors and drug resistance in cancer: the potential benefits of combination therapies of Hsp90 inhibitors and other anti-cancer drugs. *Biochemical Pharmacology*, *83*(8), 995–1004. doi:10.1016/j.bcp.2011.11.011

Luqmani, Y. A. (2005). Mechanisms of drug resistance in cancer chemotherapy. *Medical Principles and Practice : International Journal of the Kuwait University, Health Science Centre*, *14 Suppl 1*(Suppl. 1), 35–48. doi:10.1159/000086183

Mandai, M., Hamanishi, J., Abiko, K., Matsumura, N., Baba, T., & Konishi, I. (2016). Dual Faces of IFNγ in Cancer Progression: A Role of PD-L1 Induction in the Determination of Pro- and Antitumor Immunity. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *22*(10), 2329–34. doi:10.1158/1078-0432.CCR-16-0224

Massagué, J. (2004). G1 cell-cycle control and cancer. *Nature*, *432*(7015), 298–306. doi:10.1038/nature03094

Maverakis, E., Cornelius, L. A., Bowen, G. M., Phan, T., Patel, F. B., Fitzmaurice, S., Patel, F. (2015). Metastatic melanoma - a review of current and future treatment options. *Acta Dermato-Venereologica*, *95*(5), 516–24. doi:10.2340/00015555-2035

Melvold, R. W., & Sticca, R. P. (2007). Basic and Tumor Immunology: A Review. *Surg Oncol Clin N Am*, *16*, 711–735. doi:10.1016/j.soc.2007.08.003

Mhaidat, N. M., Zhang, X. D., Allen, J., Avery-Kiejda, K. A., Scott, R. J., & Hersey, P. (2007). Temozolomide induces senescence but not apoptosis in human melanoma cells. *British Journal of Cancer*, *97*(9), 1225–33. doi:10.1038/sj.bjc.6604017

Middleton, M. R., Grob, J. J., Aaronson, N., Fierlbeck, G., Tilgen, W., Seiter, S., … Thatcher, N. (2000). Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *18*(1), 158–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10623706

Miyata, Y., Nakamoto, H., & Neckers, L. (2013). The therapeutic target Hsp90 and cancer hallmarks. *Current Pharmaceutical Design*, *19*(3), 347–65. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22920906

Modi, S., Stopeck, A., Linden, H., Solit, D., Chandarlapaty, S., Rosen, N., … Hudis, C. (2011). HSP90 inhibition is effective in breast cancer: a phase II trial of tanespimycin (17-AAG) plus trastuzumab in patients with HER2-positive metastatic breast cancer progressing on trastuzumab. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *17*(15), 5132–9. doi:10.1158/1078-0432.CCR-11-0072

Muul, L. M., Spiess, P. J., Director, E. P., & Rosenberg, S. A. (1987). Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. *Journal of Immunology (Baltimore, Md. : 1950)*, *138*(3), 989–95. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3100623

Neckers, L., & Ivy, S. P. (2003). Heat shock protein 90. *Current Opinion in Oncology*, *15*(6), 419–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14624223

Neckers, L., Mimnaugh, E., & Schulte, T. W. (1999). Hsp90 as an anti-cancer target. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, *2*(3), 165–172. doi:10.1054/drup.1999.0082

Neckers, L., & Workman, P. (2012). Hsp90 molecular chaperone inhibitors: are we there yet? *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *18*(1), 64–76. doi:10.1158/1078-0432.CCR-11-1000

Niezgoda, A., Niezgoda, P., Czajkowski, R., Niezgoda, A., Niezgoda, P., & Czajkowski, R. (2015). Novel Approaches to Treatment of Advanced Melanoma: A Review on Targeted

Therapy and Immunotherapy. *BioMed Research International*, *2015*, 1–16. doi:10.1155/2015/851387

Noble, F., Mellows, T., McCormick Matthews, L. H., Bateman, A. C., Harris, S., Underwood, T. J., … Ottensmeier, C. H. (2016). Tumour infiltrating lymphocytes correlate with improved survival in patients with oesophageal adenocarcinoma. *Cancer Immunology, Immunotherapy : CII*, *65*(6), 651–62. doi:10.1007/s00262-016-1826-5

Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G. M., Apetoh, L., Perfettini, J.-L., … Kroemer, G. (2007). Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nature Medicine*, *13*(1), 54–61. doi:10.1038/nm1523

Ohba, S., Hirose, Y., Yoshida, K., Yazaki, T., & Kawase, T. (2010). Inhibition of 90-kD heat shock protein potentiates the cytotoxicity of chemotherapeutic agents in human glioma cells. *Journal of Neurosurgery*, *112*(1), 33–42. doi:10.3171/2009.3.JNS081146

Olivier, K. R., Schild, S. E., Morris, C. G., Brown, P. D., & Markovic, S. N. (2007). A higher radiotherapy dose is associated with more durable palliation and longer survival in patients with metastatic melanoma. *Cancer*, *110*(8), 1791–5. doi:10.1002/cncr.22988

Overwijk, W. W., Theoret, M. R., Finkelstein, S. E., Surman, D. R., de Jong, L. A., Vyth-Dreese, F. A., … Restifo, N. P. (2003). Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *The Journal of Experimental Medicine*, *198*(4), 569–80. doi:10.1084/jem.20030590

Pavey, S., Spoerri, L., Haass, N. K., & Gabrielli, B. (2013). DNA repair and cell cycle checkpoint defects as drivers and therapeutic targets in melanoma. *Pigment Cell & Melanoma Research*, *26*(6), 805–16. doi:10.1111/pcmr.12136

Pennisi, R., Ascenzi, P., & di Masi, A. (2015). Hsp90: A New Player in DNA Repair? *Biomolecules*, *5*(4), 2589–618. doi:10.3390/biom5042589

Persidis, A., Cancer multidrug resistance. (1999). *Nature Biotechnology*, *18*(Supp), IT18–IT20. doi:10.1038/80051

Phan, G. Q., Yang, J. C., Sherry, R. M., Hwu, P., Topalian, S. L., Schwartzentruber, D. J., … Rosenberg, S. A. (2003). Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(14), 8372–7. doi:10.1073/pnas.1533209100

Pick, E., Kluger, Y., Giltnane, J. M., Moeder, C., Camp, R. L., Rimm, D. L., & Kluger, H. M. (2007). High HSP90 expression is associated with decreased survival in breast cancer. *Cancer Research*, *67*(7), 2932–7. doi:10.1158/0008-5472.CAN-06-4511

Proia, D. A., & Kaufmann, G. F. (2015). Targeting Heat-Shock Protein 90 (HSP90) as a Complementary Strategy to Immune Checkpoint Blockade for Cancer Therapy. *Cancer Immunology Research*, *3*(6), 583–589. doi:10.1158/2326-6066.CIR-15-0057

Proia, D. A., Sang, J., He, S., Smith, D. L., Sequeira, M., Zhang, C., … Wada, Y. (2012). Synergistic activity of the Hsp90 inhibitor ganetespib with taxanes in non-small cell lung cancer models. *Investigational New Drugs*, *30*(6), 2201–2209. doi:10.1007/s10637-011-9790-6

Quirt, I., Verma, S., Petrella, T., Bak, K., & Charette, M. (2007). Temozolomide for the Treatment of Metastatic Melanoma: A Systematic Review. *The Oncologist*, *12*(9), 1114–1123. doi:10.1634/theoncologist.12-9-1114

Ramachandran, C., Samy, T. S., Huang, X. L., Yuan, Z. K., & Krishan, A. (1993). Doxorubicin-induced DNA breaks, topoisomerase II activity and gene expression in human melanoma cells. *Biochemical Pharmacology*, *45*(6), 1367–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8385463

Ramalingam, S., Goss, G., Rosell, R., Schmid-Bindert, G., Zaric, B., Andric, Z., … Fennell, D. (2015). A randomized phase II study of ganetespib, a heat shock protein 90 inhibitor, in combination with docetaxel in second-line therapy of advanced non-small cell lung cancer (GALAXY-1). *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO*, *26*(8), 1741–8. doi:10.1093/annonc/mdv220

Ramirez, M. C., & Sigal, L. J. (2004). The multiple routes of MHC-I cross-presentation. *Trends in Microbiology*, *12*(5), 204–7. doi:10.1016/j.tim.2004.03.008

Rao, A., Taylor, J. L., Chi-Sabins, N., Kawabe, M., Gooding, W. E., & Storkus, W. J. (2012). Combination therapy with HSP90 inhibitor 17-DMAG reconditions the tumor microenvironment to improve recruitment of therapeutic T cells. *Cancer Research*, *72*(13), 3196–206. doi:10.1158/0008-5472.CAN-12-0538

Rebucci, M., & Michiels, C. (2013). Molecular aspects of cancer cell resistance to chemotherapy. *Biochemical Pharmacology*. doi:10.1016/j.bcp.2013.02.017

Ribas, A., Kefford, R., Marshall, M. A., Punt, C. J. A., Haanen, J. B., Marmol, M., … Hauschild, A. (2013). Phase III Randomized Clinical Trial Comparing Tremelimumab With Standard-of-Care Chemotherapy in Patients With Advanced Melanoma. *Journal of Clinical Oncology*, *31*(5), 616–622. doi:10.1200/JCO.2012.44.6112

Richter, K., & Buchner, J. (2001). Hsp90: chaperoning signal transduction. *Journal of Cellular Physiology*, *188*(3), 281–90. doi:10.1002/jcp.1131

Rietschel, P., Wolchok, J. D., Krown, S., Gerst, S., Jungbluth, A. A., Busam, K., … Chapman, P. B. (2008). Phase II study of extended-dose temozolomide in patients with melanoma. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *26*(14), 2299–304. doi:10.1200/JCO.2007.14.5292

Ritossa, F. (1962). A new puffing pattern induced by temperature shock and DNP in drosophila. *Experientia*, *18*(12), 571–573. doi:10.1007/BF02172188

Robbins, P. F., Morgan, R. A., Feldman, S. A., Yang, J. C., Sherry, R. M., Dudley, M. E., … Rosenberg, S. A. (2011). Tumor Regression in Patients With Metastatic Synovial Cell Sarcoma and Melanoma Using Genetically Engineered Lymphocytes Reactive With NY-ESO-1. *Journal of Clinical Oncology*, *29*(7), 917–924. doi:10.1200/JCO.2010.32.2537

Robert, C., Ribas, A., Wolchok, J. D., Hodi, F. S., Hamid, O., Kefford, R., … al., et. (2014). Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. *The Lancet*, *384*(9948), 1109–1117. doi:10.1016/S0140-6736(14)60958-2

Rosenberg, S. A., Restifo, N. P., Yang, J. C., Morgan, R. A., & Dudley, M. E. (2008). Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nature Reviews Cancer*, *8*(4), 299–308. doi:10.1038/nrc2355

Rosenberg, S. A., Yang, J. C., & Restifo, N. P. (2004). Cancer immunotherapy: moving beyond current vaccines. *Nature Medicine*, *10*(9), 909–15. doi:10.1038/nm1100

Rusakiewicz, S., Semeraro, M., Sarabi, M., Desbois, M., Locher, C., Mendez, R., … Zitvogel, L. (2013). Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors. *Cancer Research*, *73*(12), 3499–510. doi:10.1158/0008-5472.CAN-13-0371

Sadelain, M., Brentjens, R., & Rivière, I. (2013). The basic principles of chimeric antigen receptor design. *Cancer Discovery*, *3*(4), 388–98. doi:10.1158/2159-8290.CD-12-0548

Sangster, T. A., Lindquist, S., & Queitsch, C. (2004). Under cover: causes, effects and implications of Hsp90-mediated genetic capacitance. *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology*, *26*(4), 348–62. doi:10.1002/bies.20020

Sankunny, M., Parikh, R. A., Lewis, D. W., Gooding, W. E., Saunders, W. S., & Gollin, S. M. (2014). Targeted inhibition of ATR or CHEK1 reverses radioresistance in oral squamous cell carcinoma cells with distal chromosome arm 11q loss. *Genes, Chromosomes & Cancer*, *53*(2), 129–43. doi:10.1002/gcc.22125

Sauvageot, C. M.-E., Weatherbee, J. L., Kesari, S., Winters, S. E., Barnes, J., Dellagatta, J., … Wen, P. Y. C. (2008). Efficacy of the HSP90 inhibitor 17-AAG in human glioma cell lines and tumorigenic glioma stem cells. *Neuro-Oncology*, *11*(2), 109–121. doi:10.1215/15228517-2008-060

Scaltriti, M., Serra, V., Normant, E., Guzman, M., Rodriguez, O., Lim, A. R., … Baselga, J. (2011). Antitumor activity of the Hsp90 inhibitor IPI-504 in HER2-positive trastuzumab-resistant breast cancer. *Molecular Cancer Therapeutics*, *10*(5), 817–24. doi:10.1158/1535-7163.MCT-10-0966

Schindler, K., & Postow, M. A. (2014). Current options and future directions in the systemic treatment of metastatic melanoma. *The Journal of Community and Supportive Oncology*. doi:10.12788/jcso.0005

Schmidt-Ullrich, R. K., Contessa, J. N., Lammering, G., Amorino, G., & Lin, P.-S. (2003). ERBB receptor tyrosine kinases and cellular radiation responses. *Oncogene*, *22*(37), 5855–65. doi:10.1038/sj.onc.1206698

Schreurs, M. W., Eggert, A. A., de Boer, A. J., Vissers, J. L., van Hall, T., Offringa, R., … Adema, G. J. (2000). Dendritic cells break tolerance and induce protective immunity against a melanocyte differentiation antigen in an autologous melanoma model. *Cancer Research*, *60*(24), 6995–7001. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11156402

Scroggins, B. T., Robzyk, K., Wang, D., Marcu, M. G., Tsutsumi, S., Beebe, K., … Neckers, L. (2007). An acetylation site in the middle domain of Hsp90 regulates chaperone function. *Molecular Cell*, *25*(1), 151–9. doi:10.1016/j.molcel.2006.12.008

Seliger, B. (2014). The link between MHC class I abnormalities of tumors, oncogenes, tumor suppressor genes, and transcription factors. *Journal of Immunotoxicology*, *11*(4), 308–10. doi:10.3109/1547691X.2013.875084

Shah, D. J., & Dronca, R. S. (2014). Latest Advances in Chemotherapeutic, Targeted, and Immune Approaches in the Treatment of Metastatic Melanoma. *Mayo Clinic Proceedings*, *89*(4), 504–519. doi:10.1016/j.mayocp.2014.02.002

Shiloh, Y. (2001). ATM and ATR: networking cellular responses to DNA damage. *Current Opinion in Genetics & Development*, *11*(1), 71–77. doi:10.1016/S0959-437X(00)00159-3

Shiloh, Y., & Lehmann, A. R. (2004). Maintaining integrity. *Nature Cell Biology*, *6*(10), 923–8. doi:10.1038/ncb1004-923

Siegel, R. L., Miller, K. D., & Jemal, A. (2016). Cancer statistics, 2016. *CA: A Cancer Journal for Clinicians*, *66*(1), 7–30. doi:10.3322/caac.21332

Siegel, R., Naishadham, D., & Jemal, A. (2013). Cancer statistics, 2013. *CA: A Cancer Journal for Clinicians*, *63*(1), 11–30. doi:10.3322/caac.21166

Slingluff, C. L., Chianese-Bullock, K. A., Bullock, T. N. J., Grosh, W. W., Mullins, D. W., Nichols, L., … Engelhard, V. H. (2006). Immunity to melanoma antigens: from self-tolerance to immunotherapy. *Advances in Immunology*, *90*, 243–95. doi:10.1016/S0065-2776(06)90007-8

Smyth, T., Van Looy, T., Curry, J. E., Rodriguez-Lopez, A. M., Wozniak, A., Zhu, M., … Wallis, N. G. (2012). The HSP90 inhibitor, AT13387, is effective against imatinib-sensitive and -resistant gastrointestinal stromal tumor models. *Molecular Cancer Therapeutics*, *11*(8), 1799–808. doi:10.1158/1535-7163.MCT-11-1046

Socinski, M. A., Goldman, J., El-Hariry, I., Koczywas, M., Vukovic, V., Horn, L., … Shapiro, G. I. (2013). A multicenter phase II study of ganetespib monotherapy in patients with genotypically defined advanced non-small cell lung cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *19*(11), 3068–77. doi:10.1158/1078-0432.CCR-12-3381

Soga, S., Akinaga, S., & Shiotsu, Y. (2013). Hsp90 inhibitors as anti-cancer agents, from basic discoveries to clinical development. *Current Pharmaceutical Design*, *19*(3), 366–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22920907

Solit, D. B., Basso, A. D., Olshen, A. B., Scher, H. I., & Rosen, N. (2003). Inhibition of Heat Shock Protein 90 Function Down-Regulates Akt Kinase and Sensitizes Tumors to Taxol. *Cancer Res.*, *63*(9), 2139–2144. Retrieved from http://cancerres.aacrjournals.org/content/63/9/2139.long

Stecklein, S. R., Kumaraswamy, E., Behbod, F., Wang, W., Chaguturu, V., Harlan-Williams, L. M., & Jensen, R. A. (2012). BRCA1 and HSP90 cooperate in homologous and non-homologous DNA double-strand-break repair and G2/M checkpoint activation. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(34), 13650–5. doi:10.1073/pnas.1203326109

Stepanova, L., Leng, X., Parker, S. B., & Harper, J. W. (1996). Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. *Genes & Development*, *10*(12), 1491–502. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8666233

Stingl, L., Niewidok, N., Müller, N., Selle, M., Djuzenova, C. S., & Flentje, M. (2012). Radiosensitizing effect of the novel Hsp90 inhibitor NVP-AUY922 in human tumour cell lines silenced for Hsp90α. *Strahlentherapie Und Onkologie : Organ Der Deutschen Röntgengesellschaft ... [et Al]*, *188*(6), 507–15. doi:10.1007/s00066-012-0080-9

Storkus, W. J., Herrem, C., Kawabe, M., Cohen, P. A., Bukowski, R. M., Finke, J. H., & Wesa, A. K. (2007). Improving immunotherapy by conditionally enhancing MHC class I presentation of tumor antigen-derived Peptide epitopes. *Critical Reviews in Immunology*, *27*(5), 485–93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18197809

Sullivan, R. J., Flaherty, K. T., Davies, H., Bignell, G. R., Cox, C., al., et, … al., et. (2013). Resistance to BRAF-targeted therapy in melanoma. *European Journal of Cancer*, *49*(6), 1297–1304. doi:10.1016/j.ejca.2012.11.019

Supino, R., Mariani, M., Capranico, G., Colombo, A., & Parmiani, G. (1988). Doxorubicin cellular pharmacokinetics and DNA breakage in a multi-drug resistant B16 melanoma cell line. *British Journal of Cancer*, *57*(2), 142–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3358904

Taipale, M., Jarosz, D. F., & Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature Reviews Molecular Cell Biology*, *11*(7), 515–528. doi:10.1038/nrm2918

Tan, D., & Lynch, H. T. (2013). *Principles of molecular diagnostics and personalized cancer medicine*. Wolters Kluwer Health/Lippincott Williams & Wilkins.

Tatokoro, M., Koga, F., Yoshida, S., Kawakami, S., Fujii, Y., Neckers, L., & Kihara, K. (2012). Potential role of Hsp90 inhibitors in overcoming cisplatin resistance of bladder cancer-

initiating cells. *International Journal of Cancer. Journal International Du Cancer*, *131*(4), 987–96. doi:10.1002/ijc.26475

Tentori, L., Lacal, P. M., & Graziani, G. (2013). Challenging resistance mechanisms to therapies for metastatic melanoma. *Trends in Pharmacological Sciences*, *34*(12), 656–66. doi:10.1016/j.tips.2013.10.003

Thompson, J. F., Scolyer, R. A., & Kefford, R. F. (n.d.). Cutaneous melanoma. *Lancet (London, England)*, *365*(9460), 687–701. doi:10.1016/S0140-6736(05)17951-3

Tokito, T., Azuma, K., Kawahara, A., Ishii, H., Yamada, K., Matsuo, N., … Hoshino, T. (2016). Predictive relevance of PD-L1 expression combined with CD8+ TIL density in stage III non-small cell lung cancer patients receiving concurrent chemoradiotherapy. *European Journal of Cancer (Oxford, England : 1990)*, *55*, 7–14. doi:10.1016/j.ejca.2015.11.020

Topalian, S. L., Hodi, F. S., Brahmer, J. R., Gettinger, S. N., Smith, D. C., McDermott, D. F., … Sznol, M. (2012). Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. *New England Journal of Medicine*, *366*(26), 2443–2454. doi:10.1056/NEJMoa1200690

Topalian, S. L., Sznol, M., McDermott, D. F., Kluger, H. M., Carvajal, R. D., Sharfman, W. H., … Hodi, F. S. (2014). Survival, Durable Tumor Remission, and Long-Term Safety in Patients With Advanced Melanoma Receiving Nivolumab. *Journal of Clinical Oncology*, *32*(10), 1020–1030. doi:10.1200/JCO.2013.53.0105

Trepel, J., Mollapour, M., Giaccone, G., & Neckers, L. (2010). Targeting the dynamic HSP90 complex in cancer. *Nature Reviews. Cancer*, *10*(8), 537–49. doi:10.1038/nrc2887

Valerie, K., & Povirk, L. F. (2003). Regulation and mechanisms of mammalian double-strand break repair. *Oncogene*, *22*(37), 5792–812. doi:10.1038/sj.onc.1206679

Vasilevskaya, I. A., Rakitina, T. V, & O'Dwyer, P. J. (2003). Geldanamycin and its 17-allylamino-17-demethoxy analogue antagonize the action of Cisplatin in human colon adenocarcinoma cells: differential caspase activation as a basis for interaction. *Cancer Research*, *63*(12), 3241–6. Retrieved from http://cancerres.aacrjournals.org/content/63/12/3241.abstract

Vasilevskaya, I. A., Rakitina, T. V, & O'Dwyer, P. J. (2004). Quantitative effects on c-Jun N-terminal protein kinase signaling determine synergistic interaction of cisplatin and 17-allylamino-17-demethoxygeldanamycin in colon cancer cell lines. *Molecular Pharmacology*, *65*(1), 235–43. doi:10.1124/mol.65.1.235

Villadangos, J. A., & Schnorrer, P. (2007). Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nature Reviews Immunology*, *7*(7), 543–555. doi:10.1038/nri2103

Wainberg, Z. A., Anghel, A., Rogers, A. M., Desai, A. J., Kalous, O., Conklin, D., … Finn, R. S. (2013). Inhibition of HSP90 with AUY922 induces synergy in HER2-amplified

trastuzumab-resistant breast and gastric cancer. *Molecular Cancer Therapeutics*, *12*(4), 509–19. doi:10.1158/1535-7163.MCT-12-0507

Waldmann, T. A. (2003). Immunotherapy: past, present and future. *Nature Medicine*, *9*(3), 269–277. doi:10.1038/nm0303-269

Walker, J. R., Corpina, R. A., & Goldberg, J. (2001). Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature*, *412*(6847), 607–614. doi:10.1038/35088000

Wang, Y., Trepel, J. B., Neckers, L. M., & Giaccone, G. (2010). STA-9090, a small-molecule Hsp90 inhibitor for the potential treatment of cancer. *Current Opinion in Investigational Drugs (London, England : 2000)*, *11*(12), 1466–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21154128

Warmerdam, D. O., & Kanaar, R. (2010). Dealing with DNA damage: Relationships between checkpoint and repair pathways. *Mutation Research/Reviews in Mutation Research*, *704*(1), 2–11. doi:10.1016/j.mrrev.2009.12.001

Weber, J. S., Yang, J. C., Atkins, M. B., & Disis, M. L. (2015). Toxicities of Immunotherapy for the Practitioner. *Journal of Clinical Oncology*, *33*(18), 2092–2099. doi:10.1200/JCO.2014.60.0379

Whitesell, L., Bagatell, R., & Falsey, R. (2003). The stress response: implications for the clinical development of hsp90 inhibitors. *Current Cancer Drug Targets*, *3*(5), 349–58. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14529386

Whitesell, L., & Lindquist, S. L. (2005). HSP90 and the chaperoning of cancer. *Nature Reviews Cancer*, *5*(10), 761–772. doi:10.1038/nrc1716

Williams, R. S., Williams, J. S., & Tainer, J. A. (2007). Mre11-Rad50-Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. *Biochemistry and Cell Biology = Biochimie et Biologie Cellulaire*, *85*(4), 509–20. doi:10.1139/O07-069

Wolchok, J. D., Kluger, H., Callahan, M. K., Postow, M. A., Rizvi, N. A., Lesokhin, A. M., … Sznol, M. (2013). Nivolumab plus Ipilimumab in Advanced Melanoma. *New England Journal of Medicine*, *369*(2), 122–133. doi:10.1056/NEJMoa1302369

Wu, R., Forget, M.-A., Chacon, J., Bernatchez, C., Haymaker, C., Chen, J. Q., … Radvanyi, L. G. (2012). Adoptive T-cell therapy using autologous tumor-infiltrating lymphocytes for metastatic melanoma: current status and future outlook. *Cancer Journal (Sudbury, Mass.)*, *18*(2), 160–75. doi:10.1097/PPO.0b013e31824d4465

Yang, J. C. (2011). Melanoma Vaccines. *The Cancer Journal*, *17*(5), 277–282. doi:10.1097/PPO.0b013e3182325f72

Ying, W., Du, Z., Sun, L., Foley, K. P., Proia, D. A., Blackman, R. K., … Banerji, U. (2012). Ganetespib, a unique triazolone-containing Hsp90 inhibitor, exhibits potent antitumor activity and a superior safety profile for cancer therapy. *Molecular Cancer Therapeutics*, *11*(2), 475–84. doi:10.1158/1535-7163.MCT-11-0755

Yoshimoto, K., Mizoguchi, M., Hata, N., Murata, H., Hatae, R., Amano, T., … Sasaki, T. (2012). Complex DNA repair pathways as possible therapeutic targets to overcome temozolomide resistance in glioblastoma. *Frontiers in Oncology*, *2*, 186. doi:10.3389/fonc.2012.00186

Younes, R., Abrao, F. C., & Gross, J. (2013). Pulmonary metastasectomy for malignant melanoma: prognostic factors for long-term survival. *Melanoma Research*, *23*(4), 307–11. doi:10.1097/CMR.0b013e3283632cbe

Yuan, J., Adamski, R., & Chen, J. (2010). Focus on histone variant H2AX: to be or not to be. *FEBS Letters*, *584*(17), 3717–24. doi:10.1016/j.febslet.2010.05.021

Zhang, J., Stevens, M. F. G., & Bradshaw, T. D. (2012). Temozolomide: Mechanisms of Action, Repair and Resistance. *Current Molecular Pharmacology*, *5*, 102–114.

Zhang, R., Luo, D., Miao, R., Bai, L., Ge, Q., Sessa, W. C., & Min, W. (2005). Hsp90-Akt phosphorylates ASK1 and inhibits ASK1-mediated apoptosis. *Oncogene*, *24*(24), 3954–63. doi:10.1038/sj.onc.1208548

Zhao, X., Bose, A., Komita, H., Taylor, J. L., Kawabe, M., Chi, N., … Storkus, W. J. (2011). Intratumoral IL-12 gene therapy results in the crosspriming of Tc1 cells reactive against tumor-associated stromal antigens. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *19*(4), 805–14. doi:10.1038/mt.2010.295

Zhou, B.-B. S., & Bartek, J. (2004). Targeting the checkpoint kinases: chemosensitization versus chemoprotection. *Nature Reviews Cancer*, *4*(3), 216–225. doi:10.1038/nrc1296

Zhou, B.-B. S., & Elledge[2], S. J. (2000). The DNA damage response: putting checkpoints in perspective. *NATURE Www.nature.com*, *408*(23).

Zhou, J., & Zhou, J. (2014). Advances and Prospects in Cancer Immunotherapy. *New Journal of Science*, *2014*, 1–13. doi:10.1155/2014/745808

Zhou, Y. J., & Binder, R. J. (2014). The heat shock protein-CD91 pathway mediates tumor immunosurveillance. *OncoImmunology*, *3*(4), e28222. doi:10.4161/onci.28222